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(54) Title: MANIPULATION OF PLANT SENESCENCE USING MODIFIED PROMOTERS

(57) Abstract: The present invention relates to methods of manipulating senescence in plants. The invention also relates to vectors useful in such methods, transformed plants with modified senescence characteristics and plant cells, seeds and other parts of such plants

Manipulation of plant senescence using modified promoters

This application is a continuation-in-part of United States Patent Application 10/363,723 entitled "Manipulation of plant senescence using an *MYB* gene promoter and cytokinin biosynthesis gene" filed March 5, 2003, which claims priority from International Patent Application PCT/AU01/01092, entitled "Manipulation of Plant Senescence Using An MYB Gene Promoter and Cytokinin Biosynthesis Genes," filed August 30 2001, which claims priority from Australian Patent Application PQ 9946, entitled "Manipulation of Plant Senescence Using An MYB Gene Promoter and Cytokinin Biosynthesis Genes," filed September 6, 2000; the contents of which are incorporated by reference herein in their entirety.

The present invention relates to methods of manipulating senescence in plants. The invention also relates to vectors useful in such methods, transformed plants with modified senescence characteristics and plant cells, seeds and other parts of such plants.

Leaf senescence involves metabolic and structural changes in cells prior to cell death. It also involves the recycling of nutrients to actively growing regions.

The regulation of plant and plant organ senescence by cytokinins has important agricultural consequences. Elevated cytokinin levels in leaves tend to retard senescence. A number of promoters have been used to regulate the expression of the *ipt* gene, whose product (isopentenyltransferase) catalyses a key step in cytokinin synthesis. However, in general, transgenic plants over-expressing the *ipt* gene have been reported to have retarded root and shoot growth, no root formation, reduced apical dominance, and reduced leaf area.

It is an object of the present invention to overcome, or at least alleviate, one or more of the difficulties or deficiencies associated with the prior art.

In one aspect, the present invention provides a method of manipulating senescence in a plant, said method including introducing into said plant a genetic construct including a modified *myb* gene promoter, or a functionally active

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fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.

In a preferred embodiment, the present invention provides a method of manipulating senescence or enhancing biomass in a plant, said method including introducing into said plant a genetic construct including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

In a further preferred embodiment, the present invention provides a vector capable of manipulating senescence or enhancing biomass in a plant, said vector including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in the biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

The manipulation of senescence relates to the plant and/or specific plant organs. Senescence of different plant organs, such as leaves, roots, shoots, stems, tubers, flowers, stolons, and fruits may be manipulated. The manipulation of plant and plant organ senescence may have important agricultural consequences, such as increased shelf life of e.g. fruits, flowers, leaves and tubers in horticultural produce and cut flowers, reduced perishability of horticultural crops, increased carbon fixation in senescence-retarded leaves leading to enhanced yields, enhanced biomass production in forage plants, enhanced seed production, etc.

"Manipulating senescence" generally relates to delaying senescence in the transformed plant relative to an untransformed control plant. However, for some applications it may be desirable to promote or otherwise modify senescence in the

plant. Senescence may be promoted or otherwise modified for example, by utilizing an antisense gene.

An effective amount of said genetic construct may be introduced into said plant, by any suitable technique, for example by transduction, transfection or transformation. By "an effective amount" is meant an amount sufficient to result in an identifiable phenotypic trait in said plant, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable amount and method of administration. See, for example, Maniatis et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

By a "modified *myb* gene promoter" is meant a promoter normally associated with a *myb* gene, which promoter is modified to delete or inactivate one or more root specific motifs and/or pollen specific motifs in said promoter.

While applicant does not wish to be restricted by theory, it is postulated that deletion or inactivation of one or more root specific motifs in said *myb* gene promoter may alleviate or overcome the problem of leaky expression of the gene encoding a cytokinin biosynthetic enzyme in plant meristems, which may affect root development in some species of plants. It is also postulated that deletion or inactivation of one or more pollen specific motifs in said *myb* gene promoter may alleviate or overcome the problem of leaky expression of the gene encoding a cytokinin biosynthetic enzyme in pollen, which may affect pollen development in some species of plants.

Preferably the modified *myb* gene promoter is a modified *myb32* gene promoter. Preferably the modified *myb* gene promoter is from *Arabidopsis,* more preferably *Arabidopsis thaliana*.

A suitable promoter which may be modified according to the present invention is described in Li et al., Cloning of three MYB-like genes from *Arabidopsis* (PGR 99-138) Plant Physiology 121:313 (1999), the entire disclosure of which is incorporated herein by reference.

By a "root specific motif" is meant a sequence of 3-7 nucleotides, preferably 4-6 nucleotides, more preferably 5 nucleotides, which directs expression of an associated gene in the roots of a plant.

Preferably the root specific motif includes a consensus sequence ATATT or AATAT.

Preferably, between one and ten, more preferably between three and eight, even more preferably between five and seven root specific motifs are deleted or inactivated, preferably deleted, in said *myb* gene promoter.

The root specific motifs may be deleted by removing individual motifs or by removing a fragment of the promoter containing one or more motifs. For example, all or part of the region between nucleotides 1 and 530, preferably between

nucleotides 110 and 530 of the *Arabidopsis thaliana myb* gene promoter may be deleted.

The deletion may be effected by cutting the nucleic acid, for example with restriction endonucleases, and ligating the cut ends to generate a promoter with a fragment removed.

For example, a modified *Arabidopsis thaliana myb* gene promoter may be prepared by removing a fragment between the *XcmI* site at positions 162-176 and the *SspI* site at positions 520-525. This generates a modified *myb* gene promoter with 6 of the 7 root specific motifs deleted. Alternatively, all 7 of the root specific motifs may be deleted, for example by deleting the region upstream of the *SspI* site at positions 520-525, or by deleting the region between nucleotides 1 and 120 together with the region between the *XcmI* site at positions 162-176 and the *SspI* site at positions 520-525.

A root specific motif may be inactivated by adding, deleting, substituting or derivatizing one or more nucleotides within the motif, so that it no longer has the preferred consensus sequence.

Preferably the modified *myb* gene promoter includes a nucleotide sequence selected from the group consisting of the sequences shown in Figures 2, 3 and 4 hereto (Sequence ID Nos: 2, 3 and 4, respectively) and functionally active fragments and variants thereof.

By a "pollen specific motif" is meant a sequence of 3-7 nucleotides, preferably 4-6 nucleotides, more preferably 4 or 5 nucleotides, which directs expression of an associated gene in the pollen of a plant.

Preferably the pollen specific motif includes a consensus sequence selected from the group consisting of TTCT and AGAA.

Preferably, between one and thirty, more preferably between three and fifteen, even more preferably between four and ten pollen specific motifs are deleted or inactivated, preferably deleted, in said *myb* gene promoter.

The pollen specific motifs may be deleted by removing individual motifs or by removing a fragment of the promoter containing one or more motifs. For example, all or part of the region between nucleotides 1 and 540, preferably between nucleotides 390 and 540 of the *Arabidopsis thaliana myb* gene promoter may be deleted.

The deletion may be effected by cutting the nucleic acid, for example with restriction endonucleases, and ligating the cut ends to generate a promoter with a fragment removed.

For example, a modified *Arabidopsis thaliana myb* gene promoter may be prepared by removing a fragment between the *XcmI* site at positions 162-176 and the *SspI* site at positions 520-525. This generates a modified *myb* gene promoter with 4 of the 23 pollen specific motifs deleted. Alternatively, 10 of the pollen specific motifs may be deleted, for example by deleting the region upstream of the *SspI* site at positions 520-525.

A pollen specific motif may be inactivated by adding, deleting, substituting or derivatizing one or more nucleotides within the motif, so that it no longer has the preferred consensus sequence.

Preferably the modified *myb* gene promoter includes a nucleotide sequence selected from the group consisting of the sequences shown in Figures 2, 3 and 4 hereto (Sequence ID Nos: 2, 3 and 4, respectively) and functionally active fragments and variants thereof.

In a further aspect of the present invention there is provided a method of enhancing biomass in a plant, said method include introducing into said plant a genetic construct including a *myb* gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.

The *myb* gene promoter or a functionally active fragment or variant thereof may be a full length *myb* gene promoter or a modified *myb* gene promoter.

The full length *myb* gene promoter may be a *myb32* gene promoter. Preferably the *myb* gene promoter is from *Arabidopsis*, more preferably *Arabidopsis thaliana*. Most preferably the *myb* gene promoter includes a nucleotide sequence selected from the group consisting of the sequence shown in Figure 1 hereto (Sequence ID No: 1) and functionally active fragments and variants thereof.

A suitable promoter is described in Li et al., Cloning of three MYB-like genes from *Arabidopsis* (PGR 99-138) Plant Physiology 121:313 (1999).

The modified *myb* gene promoter may be a modified *myb* gene promoter as hereinbefore described.

By "enhancing biomass" is meant enhancing or increasing in a transformed plant relative to an untransformed control plant a growth characteristic selected from the group consisting of total leaf area, cumulative leaf area, leaf growth dynamics (i.e. number of leaves over time), stolon length, percentage of flowering plants and seed yield per flower or per area sown. "Enhancing biomass" also includes reducing or decreasing percentage stolon death in a transformed plant relative to an untransformed control plant.

In particular, applicants have found that while the seed weight (i.e. weight of thousand seeds) of transgenic plants according to the present invention was indistinguishable from non-transgenic control plants, the total seed yield expressed on the basis of per flower or per area sown was significantly higher in the transgenic plants when compared with non-transgenic control plants of equivalent flowering intensity.

By "functionally active" in relation to a *myb* gene promoter or modified *myb* gene promoter is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of manipulating senescence in a plant by the method of the present invention. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant.

Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence to which the fragment or variant corresponds, more preferably at least approximately 90% identity, most preferably at least approximately 95% identity. Preferably the fragment has a size of at least 20 nucleotides, more preferably at least 50 nucleotides, more preferably at least 100 nucleotides, more preferably at least 200 nucleotides, most preferably at least 300 nucleotides.

By a "gene encoding an enzyme involved in biosynthesis of a cytokinin" is meant a gene encoding an enzyme involved in the synthesis of cytokines such as kinetin, zeatin and benzyl adenine, for example a gene encoding isopentyl transferase (*ipt*), or an *ipt*-like gene such as the *sho* gene (eg. from petunia). Preferably the gene is an isopentenyl transferase (*ipt*) gene or *sho* gene. In a preferred embodiment, the gene is from a species selected from the group consisting of *Agrobacterium*, more preferably *Agrobacterium tumefaciens*; *Lotus*, more preferably *Lotus japonicus*; and *Petunia*, more preferably *Petunia hybrida*.

Most preferably the gene includes a nucleotide sequence selected from the group consisting of the sequences shown in Figures 6, 8 and 10 hereto (Sequence ID Nos: 5, 7 and 9) sequences encoding the polypeptides shown in Figures 7, 9 and 11 hereto (Sequence ID Nos: 6, 8 and 10), and functionally active fragments and variants thereof.

By "functionally active" in relation to a gene encoding a cytokinin biosynthetic enzyme is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of manipulating senescence in a plant by the method of the present invention. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence, to which the fragment or variant corresponds more preferably at least approximately 90% identity, most preferably at least approximately 95% identity. Such functionally

active variants and fragments include, for example, those having conservative nucleic acid changes or nucleic acid changes which result in conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. For example, the functionally active variant may include one or more conservative nucleic acid substitutions of a sequence shown in Figure 6, 8 or 10, the resulting functionally active variant encoding an amino acid sequence shown in Figure 7, 9 or 11, respectively. Preferably the fragment has a size of at least 20 nucleotides, more preferably at least 50 nucleotides, more preferably at least 100 nucleotides, more preferably at least 500 nucleotides.

The genetic construct may be introduced into the plant by any suitable technique. Techniques for incorporating the genetic constructs of the present invention into plant cells (for example by transduction, transfection or transformation) are well known to those skilled in the art. Such techniques include *Agrobacterium* mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos, biolistic transformation and combinations thereof. The choice of technique will depend largely on the type of plant to be transformed, and may be readily determined by an appropriately skilled person.

Cells incorporating the genetic construct of the present invention may be selected, as described below, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

The methods of the present invention may be applied to a variety of plants, including monocotyledons [such as grasses (e.g. forage, turf and bioenergy grasses including perennial ryegrass, tall fescue, Italian ryegrass, red fescue, reed canary grass, big bluestem, cordgrass, napiergrass, wildrye, wild sugarcane, Miscanthus), corn, oat, wheat and barley)], dicotyledons [such as *Arabidopsis*,

tobacco, soybean, clovers (e.g. white clover, red clover, subterranean clover), alfalfa, canola, vegetable brassicas, lettuce, spinach] and gymnosperms.

In a further aspect of the present invention there is provided a vector capable of manipulating senescence in a plant, said vector including a modified *myb* gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in the biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.

In a still further aspect of the present invention there is provided a vector capable of enhancing biomass in a plant, said vector including a *myb* gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in the biosynthesis of a cytokinin, or a functionally active fragment or variant thereof.

The *myb* gene promoter or a functionally active fragment or variant thereof may be a full length *myb* gene promoter or a modified *myb* gene promoter, as described herein.

In a preferred embodiment of this aspect of the invention, the vector may further include a terminator; said promoter, gene and terminator being operably linked.

By "operably linked" is meant that said promoter is capable of causing expression of said gene in a plant cell and said terminator is capable of terminating expression of said gene in a plant cell. Preferably, said promoter is upstream of said gene and said terminator is downstream of said gene.

The vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-chromosomal and synthetic nucleic acid sequences, eg. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*; derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids

and phage DNA. However, any other vector may be used as long as it is replicable or integrative or viable in the plant cell.

The promoter, gene and terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that they are functional in the target plant cell.

A variety of terminators which may be employed in the vectors of the present invention are also well known to those skilled in the art. The terminator may be from the same gene as the promoter sequence or a different gene. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*) and the octopine synthase (*ocs*) genes.

The vector, in addition to the promoter, the gene and the terminator, may include further elements necessary for expression of the gene, in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, spacer sequences, enhancers, introns (such as the maize Ubiquitin *Ubi* intron), antibiotic resistance genes and other selectable marker genes [such as the neomycin phosphotransferase (*nptII*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinothricin acetyltransferase (*bar* or *pat*) gene], and reporter genes (such as beta-glucuronidase (GUS) gene (*gusA*)]. The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art, such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical assays (e.g. GUS assays), thin layer chromatography (TLC), northern and western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the vector are operably linked, so as to result in expression of said gene. Techniques for

operably linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

In a further aspect of the present invention there is provided a transgenic plant cell, plant, plant seed or other plant part, with modified senescence characteristics or enhanced biomass. Preferably said plant cell, plant, plant seed or other plant part includes a vector according to the present invention. Preferably the transgenic plant cell, plant, plant seed or other plant part is produced by a method according to the present invention.

The present invention also provides a transgenic plant, plant seed or other plant part derived from a plant cell of the present invention.

The present invention also provides a transgenic plant, plant seed or other plant part derived from a plant of the present invention.

The present invention will now be more fully described with reference to the accompanying examples and drawings. It should be understood, however, that the description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the figures:

Figure 1 shows the nucleotide sequence of the promoter from *myb32* gene (*atmyb*32) from *Arabidopsis thaliana* (Sequence ID No: 1), *Atmyb32* promoter sequence with MYB type, pollen specific and Root specific motifs highlighted. WAACCA (*underline/italics*) MYB1AT; GTTAGTT (**bold/box**) MYB1LEPR; CCWACC (box) MYBPZM; GGATA (*italics*) MYBST1; AGAAA (underline) POLLEN1LELAT52; ATATT (**bold**) ROOTMOTIFTAPOX1.

Figure 2 shows an *Atmyb32* promoter sequence variant (*Atmyb32xs*) with the XcmI – SspI plant sequence deleted. MYB type, pollen specific and Root specific motifs are highlighted. WAACCA (*underline/italics*) MYB1AT; GTTAGTT

(**bold/box**) MYB1LEPR; CCWACC (box) MYBPZM; AGAAA (underline) POLLEN1LELAT52; ATATT (**bold**) ROOTMOTIFTAPOX1 (Sequence ID No: 2).

Figure 3 shows an *Atmyb32* promoter variant sequence with all root motifs deleted. MYB type, pollen specific and Root specific motifs are highlighted. WAACCA (*underline/italics*) MYB1AT; CCWACC (box) MYBPZM; AGAAA (underline) POLLEN1LELAT52 (Sequence ID No: 3)

Figure 4 shows an *Atmyb32* promoter variant sequence with the *SspI* site upstream sequence deleted. MYB type, pollen specific and Root specific motifs are highlighted. WAACCA (*underline/italics*) MYB1AT; CCWACC (box) MYBPZM; AGAAA (underline) POLLEN1LELAT52 (Sequence ID No: 4).

Figure 5 shows Motifs in *Atmyb32* and *Atmyb32xs* promoter sequences.

Figure 6 shows the nucleotide sequence of the isopentenyl transferase *(ipt)* gene from *Agrobacterium tumefaciens* (Sequence ID No: 5).

Figure 7 shows the deduced amino acid sequence of the isopentyl transferase gene from *Agrobacterium tumefaciens* (Sequence ID No. 6)

Figure 8 shows the nucleotide sequence of the isopentyl transferase gene from *Lotus japonicus* (Sequence ID No. 7).

Figure 9 shows the deduced amino acid sequence of the isopentyl transferase gene from *Lotus japonicus* (Sequence ID No. 8).

Figure 10 shows the Nucleotide sequence of the cytokinin biosynthesis *Sho* gene from *Petunia hybrida* (Sequence ID No. 9)

Figure 11 shows the Deduced amino acid sequence of the cytokinin biosynthesis *Sho* gene from *Petunia hybrida* (Sequence ID No. 10).

Figure 12 shows PCR and Southern DNA analysis of *atmyb32::ipt* transgenic white clover (*Trifolium repens*) plants. a) The T-DNA region of patmyb32:ipt

showing restriction enzyme sites and location of the probes used for Southern hybridization analysis. b) Ethidium bromide stained 1% agarose gel of the PCR amplified 599 bp *nptII* and 583 bp *ipt* products. c) Southern blot hybridization with *Hind*III digested total genomic DNA isolated from PCR positive white clover plants hybridized with the *ipt* probe. d) Southern blot hybridization with *Hind*III digested total genomic DNA isolated from PCR positive white clover plants hybridized with the *nptII* probe. Lanes 1-2: two independent kanamycin resistant cv. Haifa regenerants, code: Hmi01, Hmi08 respectively; Lanes 3-12: twelve independent kanamycin resistant cv. Irrigation regenerants, codes: Imi06, Imi07, Imi08, Imi09, Imi10, Imi11, Imi12, Imi14, Imi16, Imi18 respectively; Lane C: non-transformed white clover; Lane P: positive control plasmid patmyb32ipt.

Figure 13 shows RT-PCR analysis of *ipt* mRNA expression in *atmyb32::ipt* transgenic white clover (*T. repens*) plants. Lane 1-11 are samples from 11 independent transgenic lines with corresponding plant codes as in Figure 4.8; Lane C, Control non-transformed plant; Lane P, plasmid as positive control. Total RNA was isolated from leaf tissues. Total RNA (13 μg) was used for each reverse transcription reaction and 1/5 of RT product was amplified by PCR. DNA products on the gel on the right were amplified by 2X 30 cycles intensive PCR. No reverse transcriptase was added to the corresponding RT-PCR reaction loaded into alternate lanes.

Figure 14 shows a senescence bioassay of excised leaves from *atmyb32::ipt* transgenic white clover (*T. repens*) plants. At least 30 leaves were collected from each line from similar positions on stolons of plant lines. A. The number of yellowing leaves as a fraction of the total number of excised leaves. B. Typical appearance of leaves kept on water under light for two weeks. Key to plant lines: HC, IC and Hmg, Img, non-transformed and *atmyb32::gusA* transgenic plants (cv. Haifa and Irrigation) respectively; 01 and 08, *atmyb32::ipt* transgenic Haifa lines Hmi01 and Hmi08 respectively; 11, 12, 16 and 18 *atmyb32::ipt* transgenic Irrigation lines Imi11, Imi12, Imi16 and Imi18 respectively.

Figure 15 shows A) General plant morphology, B) Normal shoot development, and C) Normal root development in *atmyb32::ipt* transgenic white clover (*T. repens*) (right) plants compared to control plants (left).

Figure 16 shows vector details for *pBMVkAtMYB32-900::ipt* [Gene: Isopentyl transferase *(IPT)*; Vector: pBMVkAtMYB32-900::ipt-nos (backbone pPZPRCS2); Selectable marker: spec; Plant selectable marker cassette: 35S::kan::35ST; Gene promoter: *AtMYB32-900*; Gene terminator: *nos.*]

Figure 17 shows nucleotide sequence of vector *pBMVkAtMYB32-900::ipt-nos* (Sequence ID No. 11)

Figure 18 shows vector details for *pBMVkAtMYB32xs::ipt-nos* [Gene: Isopentyl transferase *(IPT)*; Vector: pBMVkAtMYB32XS::ipt-nos (backbone pPZPRCS2); Selectable marker: spec; Plant selectable marker cassette: 35S::kan::35ST; Gene promoter: AtMYB32-xs; Gene terminator: nos.]

Figure 19 shows nucleotide sequence of vector *pBMVkAtMYB32xs::ipt-nos* (Sequence ID No. 12).

Figure 20 shows vector details for *pBMVhAtMYB32-900::ipt-nos* [Gene: Isopentyl transferase *(IPT)*; Vector: pBMVhAtMYB32-900::ipt-nos (backbone pPZPRCS2); Selectable marker: spec; Plant selectable marker cassette: 35S::hph::35ST; Gene promoter: AtMYB32-900; Gene terminator: nos.]

Figure 21 shows nucleotide sequence of vector *pBMVhAtMYB32-900::ipt-nos* (Sequence ID No. 13)

Figure 22 shows vector details for *pBMVhAtMYB32xs::ipt-nos* [Gene: Isopentyl transferase *(IPT)*; Vector: pBMVhAtMYB32XS::ipt-nos (backbone pPZPRCS2); Selectable marker: spec; Plant selectable marker cassette: 35S::hph::35ST; Gene promoter: AtMYB32-xs; Gene terminator: nos.]

Figure 23 shows nucleotide sequence of vector *pBMVhAtMYB32xs::ipt-nos* (Sequence ID No. 14)

Figure 24 shows vector details for *pBSubn-AtMYB32-900::ipt-nos* [Gene: Isopentyl transferase *(IPT)*; Vector: pBSubn-AtMYB32-900::ipt-nos (backbone pPZPRCS2); Selectable marker: spec; Plant selectable marker cassette: Ubi::bar::nos; Gene promoter: AtMYB32-900; Gene terminator: nos.]

Figure 25 shows nucleotide sequence of vector *pBSAtMYB32900::ipt-nos* (Sequence ID No. 15)

Figure 26 shows generation of transgenic canola containing the *pBMVhATMYB3-900::ipt-nos* and *pBMVhATMYB32xs::ipt-nos*. **A.** Canola seeds are germinated *in vitro*; **B.** Hypocotyl sections are excised from 7-day-old seedlings and inoculated with an *Agrobacterium* suspension; **C&D**. Regeneration from inoculated hypocotyl sections under hygromycin selection; **E-J.** Transgenic T_0 canola plants carrying the *pBMVhATMYB3-900::ipt-nos* and *pBMVhATMYB32xs::ipt-nos* vectors.

Figure 27 shows a process for biolistic transformation of wheat.

Figure 28 shows biolistic transformation of wheat (*Triticum aestivum* L. MPB Bobwhite 26). Donor plant production (**A & B**); zygotic embryo isolation (**C&D**); Regeneration under glufosinate selection (**E-G**); Root formation under selection (**H**); T_0 plants growing under containment glasshouse conditions for recovery of transgenic offspring (**I**).

Figure 29 shows contained field trial of transgenic white clover plants expressing chimeric Atmyb32::ipt genes.

Figure 30 shows comparative assessment of growth rates and growth dynamics of transgenic white clover plants expressing chimeric Atmyb32::ipt genes with non-transgenic control white clover plants. A) Growth Rates B) Growth Dynamics C) Growth Characteristics (after 45 days)

□ Transgenic white clover (light) ■ Non-transgenic control white clover (dark)

Figure 31 shows flowering intensity (i.e. number of ripe flower per m^2) of transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR

12, LXR 18 and LXR 11) and non-transgenic control white clover plants (i.e. WT) under contained field conditions.

Figure 32 shows seed weight (i.e. weight of thousand seeds, in grams) of transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR 12, LXR 18 and LXR 11) and non-transgenic control white clover plants (i.e. WT) under contained field conditions.

Figure 33 shows seed yield per flower (in milligrams) of transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR 12, LXR 18 and LXR 11) and non-transgenic control white clover plants (i.e. WT) under contained field conditions.

Figure 34 shows seed yield per area (in kg/ha) of transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR 12, LXR 18 and LXR 11) and non-transgenic control white clover plants (i.e. WT) under contained field conditions.

Figure 35 shows generation of transgenic alfalfa plants containing the chimeric *pBMVkATMYB3-900::ipt-nos* and *pBMVkATMYB32xs::ipt-nos* genes **A.** Petiole explants from alfalfa clones C2-3, C2-4 and 19-17 are used for inoculation with an *Agrobacterium* suspension and lead to the production of transformed embryogenic calli following selection in presence of kanamycin; **B-D**. Regeneration of transgenic alfalfa plantlets carrying chimeric genes from *pBMVkATMYB3-900::ipt-nos* and *pBMVkATMYB32xs::ipt-nos* vectors, from somatic embryos grown *in vitro*.

Figure 36 shows PCR analysis of transgenic canola plants (T1 LXR canola plants Line 4). Genomic DNA was isolated from different transgenic canola plants of T_1 LXR04 lines and subjected to PCR using primers specific for **A**. the selectable marker (*hph*) or **B**. the candidate gene of interest (*IPT*).

Figure 37 shows expression analysis of the *IPT* gene in T_1 transgenic canola plants (T1 LXR canola relative IPT leaf expression).

Figure 38 shows transgenic canola displaying delay of detached cotyledon senescence as compared to wild-type control cotyledons 7 days following detachment.

Figure 39 shows the senescence score of T_1 transgenic canola cotyledons at 7 days following detachment.

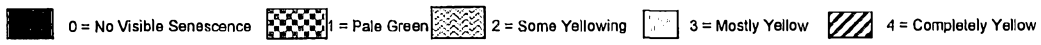

Figure 40 shows transgenic canola displaying delay of detached juvenile leaf senescence as compared to wild-type control cotyledons 14 days following detachment.

Figure 41 shows senescence score of T_1 transgenic canola juvenile first leaves at 14 days following detachment.

Figure 42 shows Southern hybridisation analysis of transgenic wheat lines. Lanes include: MW - molecular weight; WT - wild-type; Transgenic wheat lines LXR3, LXR4, LXR13, LXR16 and positive plasmid controls containing 10 and 20 pg.

Figure 43 shows expression analysis of independent T_1 transgenic wheat lines (IPT quantitative expression in wheat). Quantitative transcript values were determined in femtomoles (fmol) per microgram of RNA using a standard curve derived from plasmid DNA containing the target sequence. Samples represent high, medium and low expression classes for candidate gene *IPT*.

Figure 44 shows phenotypic variation of glasshouse grown T_1 transgenic wheat plants. **A.** T_1 LXR 13 wheat plants displaying normal phenotype as compared to null control wheat plants. **B.** T_1 LXR 04 wheat plants displaying stunted phenotype and increased flag leaf number as compared to null control wheat plants.

Figure 45 shows transgenic wheat displaying delay of leaf senescence as compared to null control leaves 7 days following detachment.

Examples

Example 1

Atmyb32 promoter sequence and promoter sequence variants

The Atmybb32 promoter sequence and variants thereof are shown in Figures 1-4.

Example 2

Cytokinin biosynthesis genes

Examples of cytokinin biosynthesis genes suitable for use in the present invention are shown in Figures 6, 8 and 10. Suitable genes also include those encoding the polypeptides shown in Figures 7, 9 and 11.

Example 3

Production of transgenic white clover plants

Transgenic white clover plants (*Trifolium repens* cv. Haifa and Irrigation) were produced by *Agrobacterium*-mediated transformation using a binary vector carrying the chimeric *atmyb32::ipt* gene (Figure 12a). The transgenic plants were screened by PCR using *ipt* and *nptII* primers (Figure 12b). *Hind*III digested genomic DNA samples subjected to Southern DNA hybridization analysis showed that the DNA fragments greater than 4.4 kb were detected in all lanes by both *ipt* and *nptII* probes, demonstrating the presence and integration of full-length T-DNA into the white clover genome (Figure 12). Transgenic lines Hmi01, Imi06, Imi11, and Imi18 (Lane 1, 3, 5, 8 and 12 respectively) appeared to have a single copy of full-length T-DNA integrated in the genome. Other transgenic lines had multiple copies of the *atmyb32::ipt* transgene.

Example 4

IPT gene expression in transgenic white clover plants

The expression of the *atmyb32::ipt* transgene in transgenic white clover (*T. repens*) plants was assessed by RT-PCR. The *ipt* mRNA was detected in leaf tissues of all *atmyb32::ipt* transgenic white clover plants examined, with varying levels of PCR products detected (Figure 13).

Example 5

Delayed detached leaf senescence in transgenic white clover plants

Experiments were performed to assess detached leaf senescence of *atmyb32::ipt* transgenic plants. Rapid yellowing was observed in detached leaves from non-transformed and *atmyb32::gusA* transgenic white clover plants of both cultivars within one week. Transgenic lines Hmi01, Hmi08, Imi16 and Imi18 showed delayed senescence while Imi11 and Imi12 showed no sign of yellowing by the end of 7 days. After two weeks, the leaves of all *atmyb32::ipt* transgenic plants were much greener than those of non-transformed and *atmyb32::gusA* control transgenic plants (Figure 14). The degree of senescence in excised leaves was in the order HC, Hmg > Hmi01 > Hmi08 for cv. Haifa, and IC and Img > Imi16 > Imi18 > Imi11 and Imi12 for cv. Irrigation. HC is Haifa untransformed control, Hmg is Haifa *atmyb32::gusA* control, IC is Irrigation untransformed control, Img is Irrigation *atmyb32::gusA* control. Hmi01, Hmi08, Imi16, Imi18, Imi11 and Imi12 are independent *atmyb32::ipt* transgenic white clover plants from the cultivar Haifa (H) and Irrigation (I), respectively.

Example 6

Plant morphology and root development in transgenic white clover plants

Normal plant morphology as well as normal shoot and normal root development was observed in *atmyb32:ipt* transgenic white clover plants (Figure 6), thus indicating that the regulated expression of the ipt gene under control of the

atmyb32 promoter did not negatively affect neither rooting nor apical dominance of the transgenic white clover plants (Table 1).

Table 1

Transformant	Cultivar	Construct	*ipt* copy No	Phenotype
Hmi01	Haifa	*Atmyb32::ipt*	1	Normal
Hmi08	Haifa	*Atmyb32::ipt*	>3	Normal
Imi06	Irrigation	*Atmyb32::ipt*	1	Normal
Imi07	Irrigation	*Atmyb32::ipt*	3	Normal
Imi09	Irrigation	*Atmyb32::ipt*	>3	Normal
Imi10	Irrigation	*Atmyb32::ipt*	>3	Normal
Imi11	Irrigation	*Atmyb32::ipt*	1	Normal
Imi12	Irrigation	*Atmyb32::ipt*	2	Normal
Imi16	Irrigation	*Atmyb32::ipt*	2	Normal
Imi18	Irrigation	*Atmyb32::ipt*	1	Normal

Normal plant morphology and normal rooting was observed in ten independent *atmyb32::ipt* transgenic white clover lines analysed. Estimated *ipt* gene copy numbers in the ten independent *atmyb32::ipt* transgenic white clover lines are shown.

Example 7

Generation of vectors for plant transformation

Four binary vectors have been generated for *Agrobacterium*-mediated transformation of plants (Figures 16-19). Each vector has a pPZP200 vector backbone (Hajdukiewicz *et al.*, 1994) and contains either chimeric *Atmyb32-900::ipt-nos* or *Atmyb32-xs::ipt-nos* with or without a chimeric *35S::nptII-35st* or *35S::hph-35st* selectable marker cassettes.

One transformation vector has been constructed for biolistic transformation (Figures 20 and 21). The transformation vector contains chimeric *Atmyb32-900::ipt-35st* with a chimeric *Ubi::bar-nos* selectable marker cassette.

The *Atmyb32* promoter, promoter variant *Atmyb32xs*, the isopentyl transferase gene and terminators *35st* and *nos* were amplified by PCR using Gateway™ (Invitrogen) adapted primers and cloned into a pDONR221 entry vectors. These were subsequently cloned using recombination into destination vectors containing the conventionally cloned selectable marker cassettes. All vectors were fully sequenced following strict quality assurance protocols.

Example 8

***Agrobacterium*-mediated transformation of canola (*Brassica napus*)**

Binary vectors *pBMVhATMYB3-900::ipt-nos* (Figure 20) and *pBMVhATMYB32xs::ipt-nos* (Figure 22) containing chimeric *ipt* genes under control of *Atmyb32* promoter (Figure 1) and Atmyb32xs variant promoter sequence with deleted root-specific motifs (Figure 2) were used for *Agrobacterium*-mediated transformation of *Brassica napus* hypocotyl segments (Figure 26).

Brassica napus seeds are surface sterilised in 70% ethanol for 2 minutes, washed 3 times in sterile water then further surface sterilised in a solution containing 1% (w/v) Calcium hypochlorite and 0.1 % (v/v) Tween 20 for 30 minutes. The seeds are washed at least 3 times in sterile water and planted in 120 ml culture vessels containing a solidified germination medium containing 1x Murashige and Skoog (Murashige and Skoog *Physiol. Plant, 15*: 473-497, 1962) macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 2% (w/v) sucrose at a pH of 5.8 with the addition of 4 g/L Gelrite. The vessels are incubated at 25 °C under 16h light/8h dark conditions for 7 days to encourage germination.

After 7 days, seedlings of *Brassica napus* (whole seedlings) are transferred to a liquid medium consisting of 1x Murashige and Skoog macronutrients, 1x

micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 3% (w/v) sucrose at a pH of 5.8. Seedlings are grouped together and the roots and cotyledons removed prior to cutting the hypocotyls into 7-10mm sections and plating on 9 X 1.5 cm petri dishes containing a preconditioning medium consisting of 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 3% (w/v) sucrose at a pH of 5.8 solidified with 6.4 g/l Bacto-Agar.

Hypocotyl sections are cultured for 24 hours prior to inoculation with an *Agrobacterium* suspension OD_{600} = 0.2 for 30 minutes consisting of 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 100 μM Acetosyringone, 3% (w/v) sucrose at a pH of 5.8.

Following inoculation, hypocotyl sections are blotted on sterile paper towels and transferred to 9 x 1.5 cm petri dishes containing 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 100 μM Acetosyringone, 1 mg/L 2,4-D, 3% (w/v) sucrose at a pH of 5.8 solidified with 8 g/l Bacto-Agar. Explants are incubated at 25°C under 16h light/8h dark conditions for 72 hours for co-cultivation.

Following co-cultivation, 20-30 hypocotyl explants are transferred to 9 X 1.5 cm petri dishes containing a solidified selection medium consisting of 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 1 mg/L 2,4-D, 3% (w/v) sucrose at a pH of 5.8 solidified with 8 g/l Bacto-Agar, supplemented with 250 mg/l timentin and 10 mg/l hygromycin to select for hygromycin-resistant shoots. Plates are incubated at 25°C under 16h light/8h dark conditions.

After 7 days hypocotyl explants are transferred to 9 X 2.0cm petri dishes containing a solidified regeneration media consisting of 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 1 mg/L 2,4-D, 3% (w/v) sucrose at a pH of 5.8 solidified with 8 g/l Bacto-Agar, supplemented with 4 mg/l BAP, 2 mg/l Zeatin, 5 mg/l Silver Nitrate,

250 mg/l timentin and 10 mg/l hygromycin. Plates are incubated under direct light at 25°C under fluorescent light conditions (16 hr light/8 hr dark photoperiod; 55 μmol m^{-2} sec^{-1}) for 4 weeks to encourage shoot development.

Regeneration is monitored weekly and hypocotyl explants transferred to fresh 9 X 2.0cm petri dishes containing solidified regeneration media, RM supplemented with 4 mg/l benzyladenine, 2 mg/l zeatin, 5 mg/l silver nitrate, 250 mg/l timentin and 10 mg/l hygromycin for 6-8 weeks to encourage shoot development.

Hygromycin-resistant (Hyg^r) shoots are transferred to 120 ml vessels containing solidified root induction medium, RIM1, consisting of 1x Murashige and Skoog macronutrients, 1x micronutrients and B5 organic vitamins, supplemented with 500 mg/L MES, 1 mg/L 2,4-D, 1% (w/v) sucrose at a pH of 5.8 solidified with 8 g/l Bacto-Agar supplemented with 250 mg/l timentin. Shoots are incubated under direct fluorescent light at 25°C (16 hr light/8 hr dark photoperiod; 55 μmol m^{-2} sec^{-1}) to encourage shoot elongation and root development over 4-5 weeks. All Hyg^r shoots with developed shoot and root systems are transferred to soil and grown under glasshouse conditions.

Example 9

Biolistic transformation of wheat (*Triticum aestivum* L.)

Transformation vectors containing chimeric *ipt* genes under control of *Atmyb32* promoter (Figure 1) and Atmyb32xs variant promoter sequence with deleted root-specific motifs (Figure 2) were used for biolistic transformation of wheat (*Triticum aestivum* L. MPB Bobwhite 26). A representative vector is shown in Figure 24. A schematic of the procedure for biolistic transformation of wheat is outlined in Figure 27. The transformation procedure includes the following steps:

Step 1 (Donor Plant Production):

Triticum aestivum (Bobwhite 26) seed is used for the production of donor plant material. Wheat plants are grown in a nursery mix consisting of composted pine bark, perlite and vermiculite, with five plants per pot to a maximum pot size of 20

cm. Plants are kept under glasshouse conditions at approximately 22-24° C for 12-16 weeks (Figure 28A). Once the first spike emerges from the flag leaf, plants are tagged and embryos collected from the tallest heads 12-15 days post anthesis.

Step 2 (Day 1)

Spikes at the desired stage of development are harvested (Figure 28B). Caryopsis are removed from the spikes and surface sterilised for 20 minutes in a 0.8% (v/v) NaOCl solution and rinsed at least four times in sterile distilled water.

Embryos up to 10 mm in length are aseptically excised from each caryopsis (removing the axis) using a dissecting microscope and cultured axial side down on an osmotic medium (E3maltose) consisting of 2x Murashige and Skoog (1962) macronutrients, 1x micronutrients and organic vitamins, 40 mg/L thiamine, 150 mg/L L-asparagine, supplemented with 15% (w/v) maltose, 0.8% (w/v) Sigma-agar and 2.5 mg/L 2,4-D (Figure 28C&D). Embryos are cultured on 60 mm x 15 mm clear polypropylene petrie dishes with 15 mL of media. Culture plates are incubated at 24 °C in the dark for 4 hours prior to bombardment. Embryos are bombarded using a BioRad PDS1000 gene gun at 900 psi and at 6 cm with 1 μg of vector plasmid DNA precipitated onto 0.6 μm gold particles. Following bombardment, embryos are incubated overnight in the dark on the osmotic media.

Step 3 (Day 2):

Embryos are transferred to a callus induction medium (E3calli) consisting of 2x Murashige and Skoog (1962) macronutrients and 1x micronutrients and organic vitamins, 40 mg/L thiamine, 150 mg/L L-asparagine, supplemented with 6% (w/v) sucrose, 0.8% (w/v) Sigma-agar and 2.5 mg/L 2,4-D. Embryos are cultured for two weeks at 24 °C in the dark.

Step 4 (Day16):

After 2 weeks of culture on E3calli, embryos have produced embryogenic callus and are subcultured onto a selection medium (E3Select) consisting of 2x Murashige and Skoog (1962) macronutrients and 1x micronutrients and organic vitamins, 40 mg/L thiamine, 150 mg/L L-asparagine, supplemented with 2% (w/v)

sucrose, 0.8% (w/v) Sigma-agar, 5 mg/L of D,L phosphinothricin (PPT) and no plant growth regulators (Figure 28E-G). Cultures are incubated for further 14 days on E3Select at 24 °C in the light and a 12-hour photoperiod.

Step 5 (Day 30):

After 14 days culture on E3Select, embryogenic callus is sub-cultured onto fresh E3Select for a further 14 days (Figure 28E-G).

Step 6 (Day 44):

After about 4 weeks on E3Select, developing plantlets are excised from the embryonic callus mass and grown for a further three weeks in 65 mm X 80 mm or 65 mm x 150 mm polycarbonate tissue culture vessels containing root induction medium (RM). Root induction medium consists of 1x Murashige and Skoog (1962) macronutrients, micronutrients and organic vitamins, 40 mg/L thiamine, 150 mg/L L-asparagine, supplemented with 2% (w/v) sucrose, 0.8% (w/v) Sigma-agar, and 5 mg/L of PPT (Figure 28H). Remaining embryogenic callus is sub-cultured onto E3Select for another 14 days.

Step 7 (Day 65+):

Regenerated plantlets surviving greater than 3 weeks on RM with healthy root formation are potted into a nursery mix consisting of peat and sand (1:1) and kept at 22-24 °C with elevated humidity under a nursery humidity chamber system (Figure 28). After two weeks, plants are removed from the humidity chamber and hand watered and liquid fed Aquasol™ weekly until maturity. The T_0 plants are sampled for genomic DNA and molecular analysis. T_1 seed is collected and planted for high-throughput Q-PCR analysis (Figure 28J).

Example 10

Agronomic performance of transgenic white clover plants

The agronomic performance of *atmyb32::ipt* transgenic white clover (*Trifolium repens*) plants, relative to that of non-transgenic control white clover plants, was

evaluated under environmentally controlled growth chamber conditions and in contained field trials (Figure 29).

Transgenic white clover plants expressing chimeric Atmyb32::ipt genes assessed under controlled growth chamber conditions revealed a significantly enhanced biomass accumulation and reductions in manifestations of senescence, when compared with non-transgenic control white clover plants (Figure 30). The transgenic white clover plants expressing chimeric Atmyb32::ipt genes showed enhanced total leaf area, increased cumulative leaf area, higher leaf growth dynamics (i.e. number of leaves over time), higher stolon length and increased % flowering plants as well as reduced stolon senescence and death compared with non-transgenic control white clover plants (Figure 30A-C).

The seed yield performance of 3 independent *atmyb32::ipt* expressing transgenic white clover plants (i.e. LXR 12, LXR 18 and LXR 11) was also comparatively assessed with non-transgenic control plants (i.e. wild type, WT) under contained field conditions. Two independent *atmyb32::ipt* expressing transgenic white clover plants (i.e. LXR 12 and LXR 18) with indistinguishable flowering intensity (i.e. number of ripe flowers per m^2) to the non-transgenic control plant (i.e. WT) were selected for field evaluation (Figure 31).

While the seed weight (i.e. weight of thousand seeds) of transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR 12, LXR 18 and LXR 11) was indistinguishable from non-transgenic control white clover plants (i.e. WT) (Figure 32), the total seed yield expressed on the basis of per flower (Figure 33), and per area sown (Figure 34) was doubled in transgenic white clover plants expressing chimeric Atmyb32::ipt genes (i.e. LXR 12 and LXR 18) when compared with non-transgenic control white clover plants of equivalent flowering intensity (i.e. WT).

Example 11

Agrobacterium-mediated transformation of alfalfa (*Medicago sativa*)

The binary vector *pBMVkATMYB32xs::ipt-nos* (Figure 18) containing chimeric *ipt* genes under control of Atmyb32xs variant promoter sequence with deleted root-specific motifs (Figure 2) was used for *Agrobacterium*-mediated transformation of *Medicago sativa* petiole explants from highly-regenerable alfalfa (*M. sativa*) clones C2-3, C2-4 and 19-17 (Figure 35).

Following co-cultivation with *Agrobacterium tumefaciens* strain LBA 4404 harbouring the binary vector *pBMVkATMYB32xs::ipt-nos,* the alfalfa explants were washed with medium containing cefotaxime and used for induction of embryogenic callus under selective medium containing 25 mg/l kanamycin. Transgenic embryogenic alfalfa calli were recovered and allowed to regenerate transgenic alfalfa shoots, which were transferred on rooting medium leading to the recovery of transgenic alfalfa plants expressing chimeric *ipt* genes under control of Atmyb32xs variant promoter (Figure 35).

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

Example 12

Production of transgenic canola plants

Transgenic canola plants (*Brassica napus*) were produced by *Agrobacterium*-mediated transformation using binary vectors (Figures 20 and 22) carrying the chimeric *atmyb32::ipt* gene. The genetic modification has been characterised for the presence of the candidate gene (*IPT*) or the selectable marker (*hph*) using PCR at the T_1 generation (Figures 36).

Figure 36 illustrates PCR analysis of transgenic canola plants. Genomic DNA was isolated from different transgenic canola plants of T_1 LXR04 lines and subjected to PCR using primers specific for the selectable marker (*hph* gene) or the candidate gene of interest (*IPT*). In Figure 36A *hph* specific primers were used to amplify a product from genomic DNA and were visualised on an agarose gel. Figure 36B demonstrates the use of *IPT* specific primers to amplify genomic DNA using a fluorescent PCR method. The primers are specific for the target sequence which results in detectable fluorescence that is inversely proportional to the amount of accumulated PCR product.

Example 13

***IPT* gene expression in transgenic canola plants**

The expression of the *atmyb32::ipt* transgene in transgenic canola was assessed using a fluorescent RT-PCR method specific for the target sequence (Figure 37). The *IPT* mRNA was detected in tissues and relative expression levels were compared among lines and with null controls. Null controls are progeny lines that have undergone the transformation process but do not contain target sequences after crossing.

Example 14

Delayed detached leaf senescence in transgenic canola plants

Experiments were performed to assess detached leaf senescence of *atmyb32::ipt* transgenic plants. Figures 38 to 41 indicate detached senescence assay data associated with expression of the candidate gene in canola. Assays for detached cotyledons and leaves were conducted to induce aging and asses the senescence phenotype of transformed canola as compared to wild-type controls. At day 7 and 14 of the detached senescence assays the progress of senescence was qualitatively scored for each tissue sample as either 0 - no possible signs of senescence; 1 - first visible signs of senescence with a paling of the green colour: 2 - further progression of senescence with yellowing in colour becoming noticeable; 3 - the tissue was mostly yellow in colour, however a pale green colour

remained evident; 4- progression to completely yellow in colour; 5 - yellow in colour with some bleaching and patches of necrosis (Figures 39 and 41).

Example 15

Production of transgenic wheat plants

Genetic transformation of wheat was based on biolistic transformation of zygotic embryos from *Triticum aestivum* L Bobwhite 26 wheat line as outlined in Figures 27 and 28.

The chimeric *atmyb32::ipt* gene was inserted into the wheat genome by particle bombardment using whole plasmids so vector backbone sequences may also be incorporated into the genome (Figure 24).

The transformation vector has been fully sequenced (Figure 25). The genetic modification has been characterised for the presence of the candidate gene by Southern analysis at the T_1 generation (Figure 42).

Figure 42 illustrates Southern hybridisation analysis of transgenic wheat plants. Genomic DNA was isolated from different T_1 lines of transgenic wheat plants and digested with a restriction enzyme to determine candidate gene copy number. The control is non-transformed wild-type *Triticum aestivum* 'Bobwhite 26'. Digests were electrophoresed, transferred to nylon membrane and probed with a full-length DIG labelled *IPT* gene, as a probe. A range of copy numbers was observed.

Example 16

IPT gene expression in transgenic wheat plants

RNA was extracted from young leaf tissue of glasshouse grown transgenic T_1 wheat plants containing the *IPT* gene driven by the AtMYB32 promoter and first strand cDNA prepared.

Quantitative expression of the transgene was determined using a probe based qRT-PCR method for the target sequence. Representative examples of high, medium and low expressing lines for each of the constructs are presented in Figure 43. A primer /probe set designed to the endogenous sucrose synthase gene was also used as a control. All amplification plots of the control gene began with in one cycle of each other indicating differences in the level of detection of the GMOs is due to variation in expression.

Both the PCR primers and probe are specific for the target sequence which results in detectable fluorescence that is proportional to the amount of accumulated PCR product. Serially diluted plasmid DNA containing the target sequence being detected was employed to create a standard curve for quantification.

Example 17

Plant morphology in transgenic wheat plants

Differences in growth characteristics were observed in the glasshouse within and among transgenic wheat lines. The phenotypes predominantly observed among T_1 wheat plants included stunted plant height, tillering intensity, leaf number, as well as vegetative biomass (Figure 44).

Example 18

Delayed detached leaf senescence in transgenic wheat plants

A detached leaf assay was used to asses induced aging and the senescence phenotype of transformed wheat leaves as compared to null controls (Figure 45). Null controls are progeny lines that have undergone the transformation process but do not contain target sequences after crossing.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

Documents cited in this specification are for reference purposes only and their inclusion is not an acknowledgement that they form part of the common general knowledge in the relevant art.

Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method of manipulating senescence or enhancing biomass in a plant, said method including introducing into said plant a genetic construct including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

2. A method according to claim 1, wherein one or more root specific motifs are deleted or inactivated and wherein each of said root specific motifs includes a consensus sequence ATATT or AATAT.

3. A method according to claim 1 or 2, wherein one or more pollen specific motifs are deleted or inactivated and wherein each of said pollen specific motifs includes a consensus sequence TTCT or AGAA.

4. A method according to any one of claims 1 to 3, wherein said myb gene promoter includes a nucleotide sequence of SEQ ID No: 1, or a functionally active fragment or variant thereof having at least 95% identity to the part of SEQ ID No: 1 to which the fragment or variant corresponds and having a size of at least 300 nucleotides.

5. A method according to claim 1, wherein said myb gene promoter includes a nucleotide sequence selected from the group consisting of SEQ ID Nos: 2, 3 and 4, and functionally active fragments and variants thereof.

6. A method according to claim 5, wherein said functionally active fragments and variants have at least 95% identity to the part of SEQ ID No: 2, 3 or 4 to which the fragment or variant corresponds and have a size of at least 300 nucleotides.

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7. A method according to any one of claims 1 to 6, wherein said gene encoding an enzyme involved in biosynthesis of a cytokinin is an isopentenyl transferase (ipt) or sho gene, or a functionally active fragment or variant thereof.

8. A method according to claim 7, wherein said gene encoding an enzyme involved in biosynthesis of a cytokinin includes a nucleotide sequence selected from the group consisting of Sequence ID Nos: 5, 7 and 9, and sequences encoding the polypeptides having a sequence of SEQ ID Nos: 6, 8 or 10.

9. A method according to any one of claims 1 to 8, wherein said genetic construct is introduced into said plant by Agrobacterium-mediated or biolistic transformation of plant cells.

10. A method according to claim 9, wherein plant cells incorporating the genetic construct are selected and then cultured to regenerate transformed plants.

11. A vector capable of manipulating senescence or enhancing biomass in a plant, said vector including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in the biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

12. A vector according to claim 11, further including a terminator; said promoter, gene and terminator being operatively linked.

13. A vector according to claim 11 or 12, wherein said one or more root specific motifs are deleted or inactivated in said modified myb gene promoter and wherein each of said root specific motifs includes a consensus sequence ATATT or AATAT.

14. A vector according to any one of claims 11 to 13, wherein one or more pollen specific motifs are deleted or inactivated in said modified myb promoter and wherein each of said pollen specific motifs includes a consensus sequence TTCT or AGAA.

15. A vector according to any one of claims 11 to 14, wherein said myb gene promoter includes a nucleotide sequence of SEQ ID No: 1, or a functionally active fragment or variant thereof having at least 95% identity to the part of SEQ ID No: 1 to which the fragment or variant corresponds and having a size of at least 300 nucleotides.

16. A vector according to claim 11, wherein said myb gene promoter includes a nucleotide sequence selected from the group consisting of Sequence ID Nos: 2, 3 and 4, and functionally active fragments and variants thereof.

17. A vector according to claim 16, wherein said functionally active fragments and variants have at least 95% identity to the part of SEQ ID No: 2, 3 or 4 to which the fragment or variant corresponds and have a size of at least 300 nucleotides.

18. A vector according to any one of claims 11 to 17, wherein said gene encoding an enzyme involved in biosynthesis of a cytokinin is an isopentenyl transferase (ipt) or sho gene, or a functionally active fragment or variant thereof.

19. A vector according to claim 18, wherein said gene encoding an enzyme involved in biosynthesis of a cytokinin includes a nucleotide sequence selected from the group consisting of SEQ ID Nos: 5, 7 and 9, and sequences encoding polypeptides having a sequence of SEQ ID Nos: 6, 8 or 10.

20. A transgenic plant cell, plant, plant seed or other plant part, with modified senescence characteristics, said plant cell, plant, plant seed or other plant part including a genetic construct including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding

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an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

21. A transgenic plant cell, plant, plant seed or other plant part, according to claim 20, produced by a method including introducing into said plant a genetic construct including a modified myb gene promoter, or a functionally active fragment or variant thereof, operatively linked to a gene encoding an enzyme involved in biosynthesis of a cytokinin, or a functionally active fragment or variant thereof, wherein one or more root specific motifs and/or pollen specific motifs are deleted or inactivated in said modified myb gene promoter.

22. A transgenic plant, plant seed or other plant part derived from a plant cell or plant according to claim 20 or 21.

23. A method of manipulating senescence in a plant, said method including introducing into said plant a genetic construct including a modified myb gene promoter, having between one and ten root specific motifs deleted or inactivated, said root specific motifs having a consensus sequence ATATT or AATAT, or having between one and thirty pollen specific motifs deleted or inactivated, said pollen specific motifs having a consensus sequence TTCT or AGAA; said modified myb gene promoter being operatively linked to an ipt or sho gene.

24. A method according to claim 1 or 23, a vector according to claim 11, or a transgenic plant cell, plant, plant seed or other plant part according to claim 20, substantially as hereinbefore described with reference to any one of the figures or examples.

```
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Figure 1.

```
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421 aacggagccc actagccact ccttcaaacg tgtcttacca actctcttct agaaacaaat
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541 ctctccataa agccctaatt tcttcatcac aagaatcaga agaagaaa
```

Figure 2.

```
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361 taggcttcac cttcctcttc caaccctctct ctctctctct ctctcttttt ctcaaaccat
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```

Figure 3.

```
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 61 tggtttcaca cctacaacta ggctaataag taaataagta gacaactaga ctcaggtttg
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181 tgttgcagtt tctagttttg atacaaacaa acaaaaacac aatttaatct tagattaaaa
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301 tagaaacaaa ttaggcttca ccttcctctt ccaaccтctc tctctctctc tctctctttt
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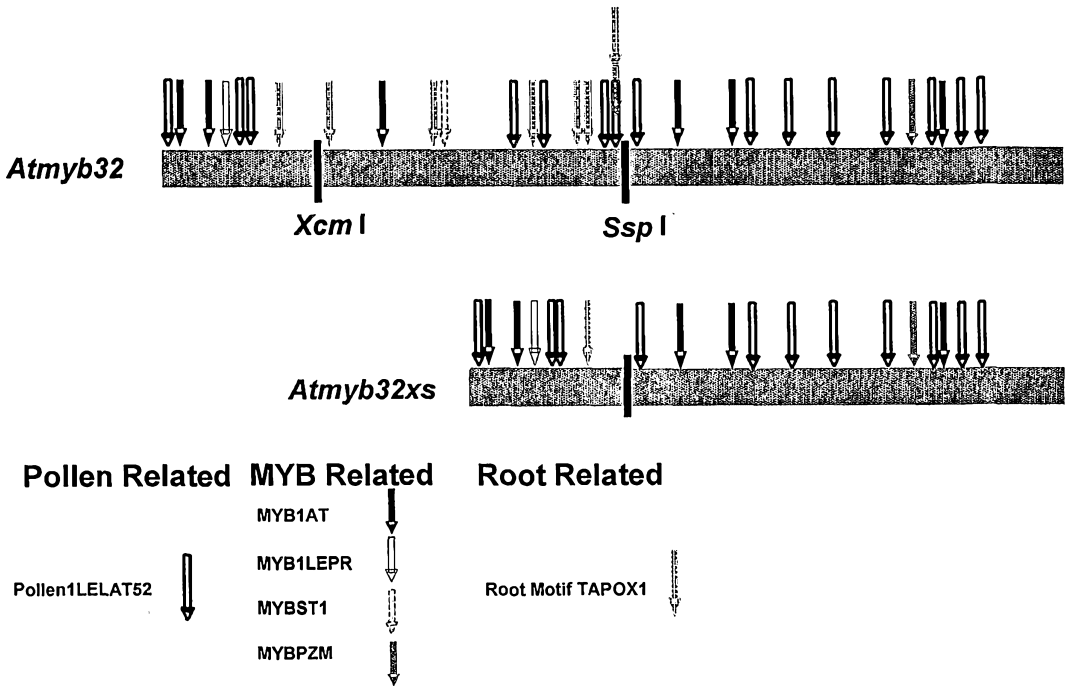
Figure 4.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Xcm I
Ssp I
Atmyb32xs
Pollen Related
MYB Related
Root Related
MYB1AT
MYB1LEPR
MYBST1
MYBPZM
Pollen1LELAT52
Root Motif TAPOX1

Figure 5.

```
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601 aagttgatta atgggatcgc tcaggagtat ttcatccatg cgcgccaaca ggaacagaaa
661 ttcccccaag ttaacgcagc cgctttcgac ggattcgaag gtcatccgtt cggaatgtat
721 tag
```

Figure 6.

```
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121 IRHKLPDQET FMKAAKARVK QMLHPAAGHS IIQELVYLWN EPRLRPILKE IDGYRYAMLF
181 ASQNQITADM LLQLDANMEG KLINGIAQEY FIHARQQEQK FPQVNAAAFD GFEGHPFGMY
241 *
```

Figure 7.

```
   1 atgtccatct caatgctaat gtgcagacta agacaaccct taataaacgt tccctgcagt
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Figure 8.

```
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181 EELRPFFNAN GDYSRGIRRA IGVPEFDEYF RREGFADEET RKLLLERAVR EMKVNTCKLA
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301 VNVVSGGFRV PAGSTESVMA AATC*
```

Figure 9.

```
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Figure 10.

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181 RFDPDFNPDS NPVHFISNEL RYNCCFIWVD VLNPVLNEYL DKRVDEMMNS GMYEELEQFF
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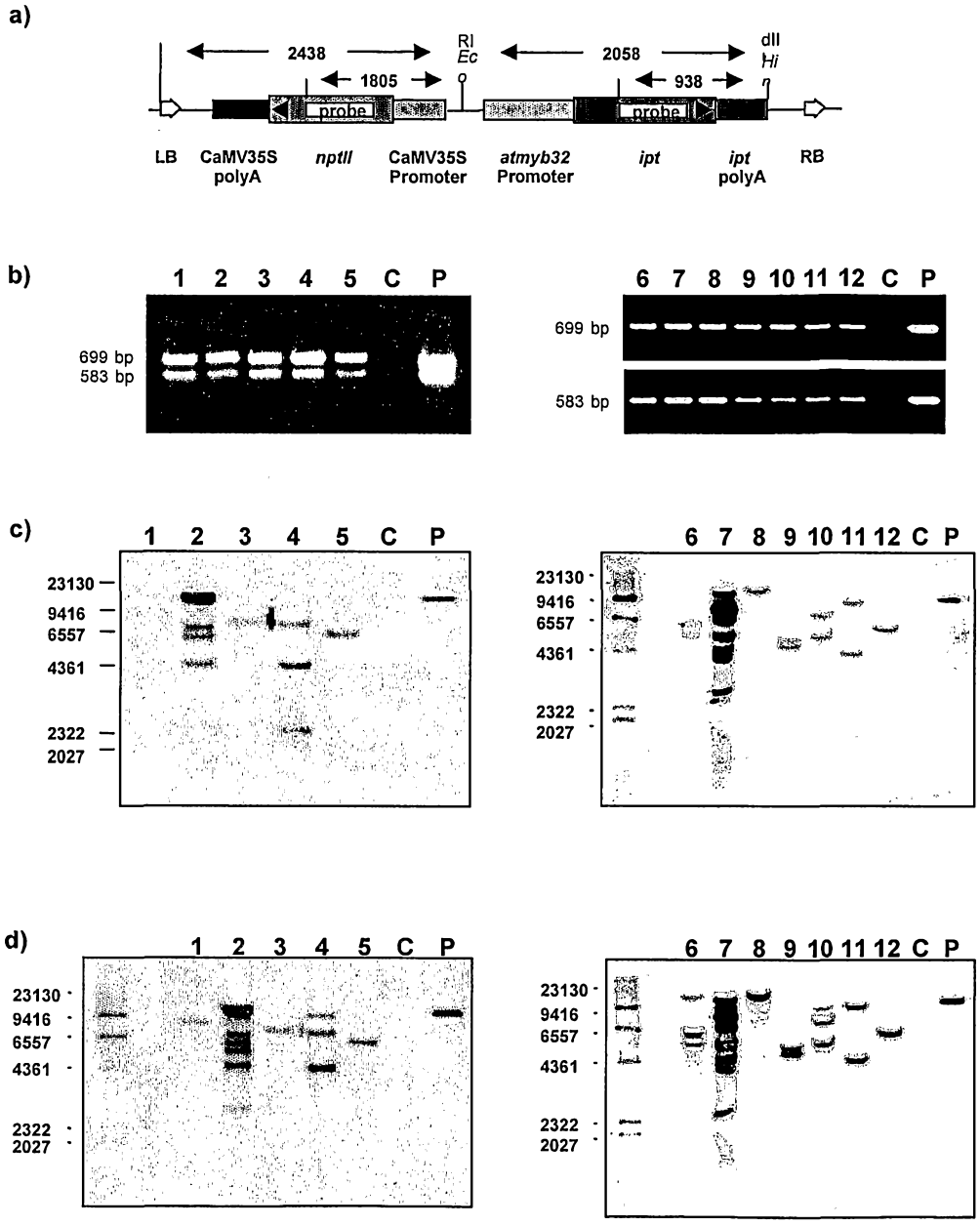
Figure 11.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probe
LB
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atmyb32 Promoter
ipt
ipt polyA
RB
b)
1 2 3 4 5 C P
699 bp
583 bp
6 7 8 9 10 11 12 C P
699 bp
583 bp
c)
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6557
4361
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d)
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Figure 12.

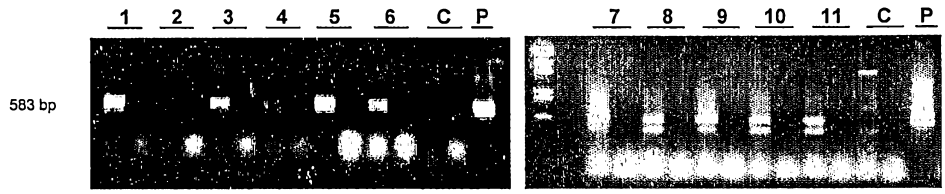


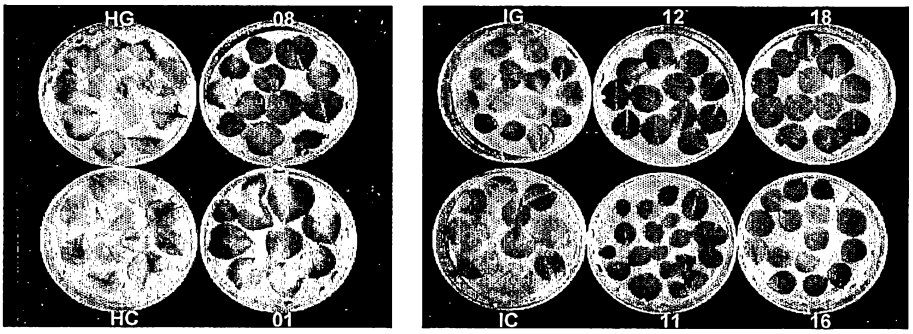
Figure 13.

A

Genotype	HC	Hmg	01	08
Week1	8/30	9/31	3/30	0/29
Week2	21/30	19/31	9/30	4/29

Genotype	HC	Hmg	11	12	16	18
Week1	9/30	5/36	0/39	0/36	4/39	1/36
Week2	19/30	15/36	1/39	1/36	17/39	5/36

B



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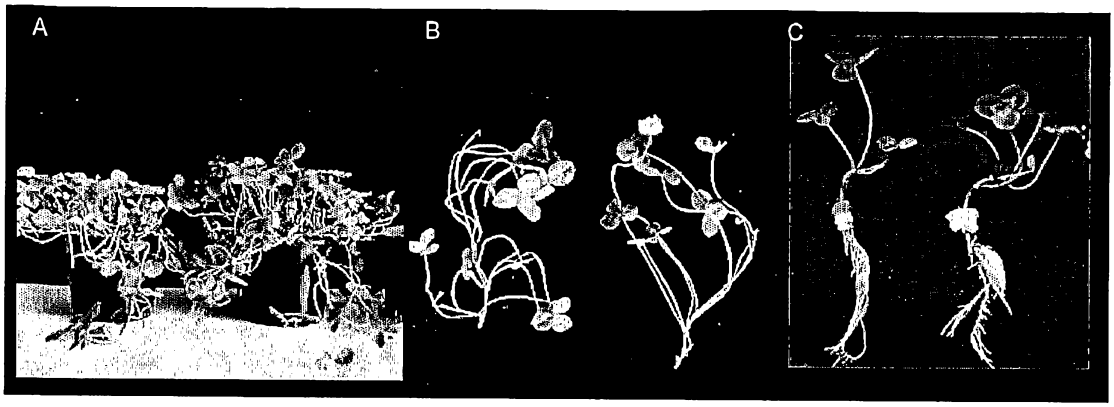

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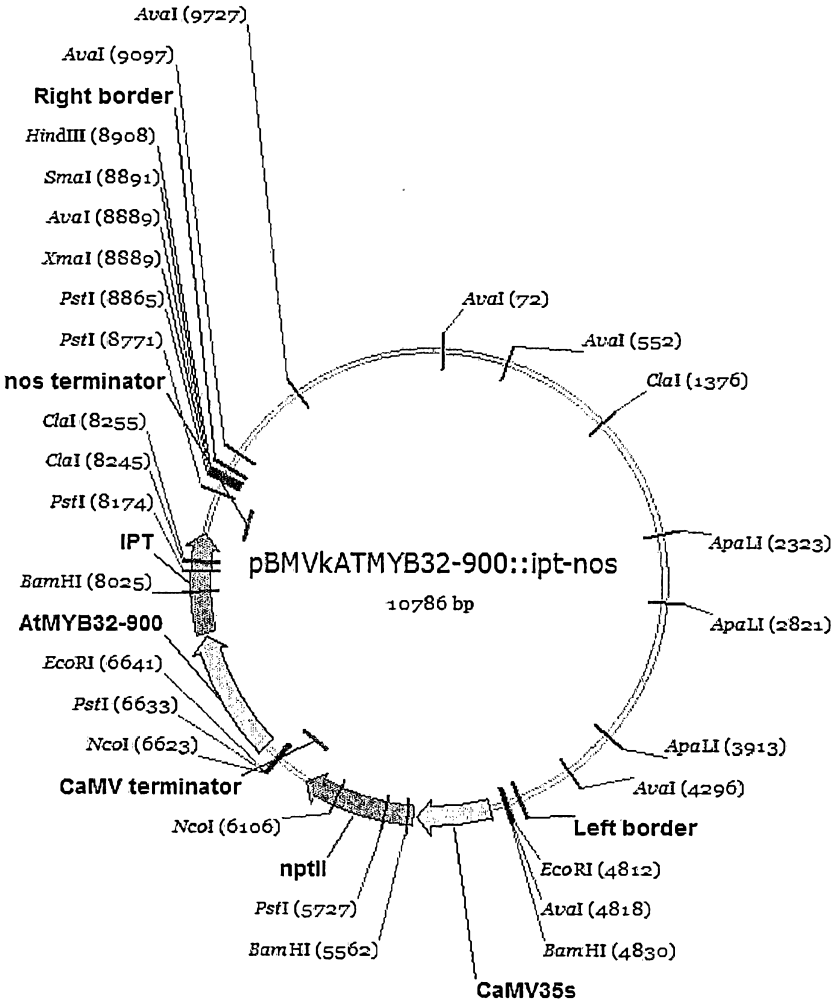
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PstI (8771)
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ClaI (8255)
ClaI (8245)
PstI (8174)
IPT
BamHI (8025)
AtMYB32-900
EcoRI (6641)
PstI (6633)
NcoI (6623)
CaMV terminator
NcoI (6106)
nptII
PstI (5727)
BamHI (5562)
AvaI (72)
AvaI (552)
ClaI (1376)
ApaLI (2323)
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Figure 16.

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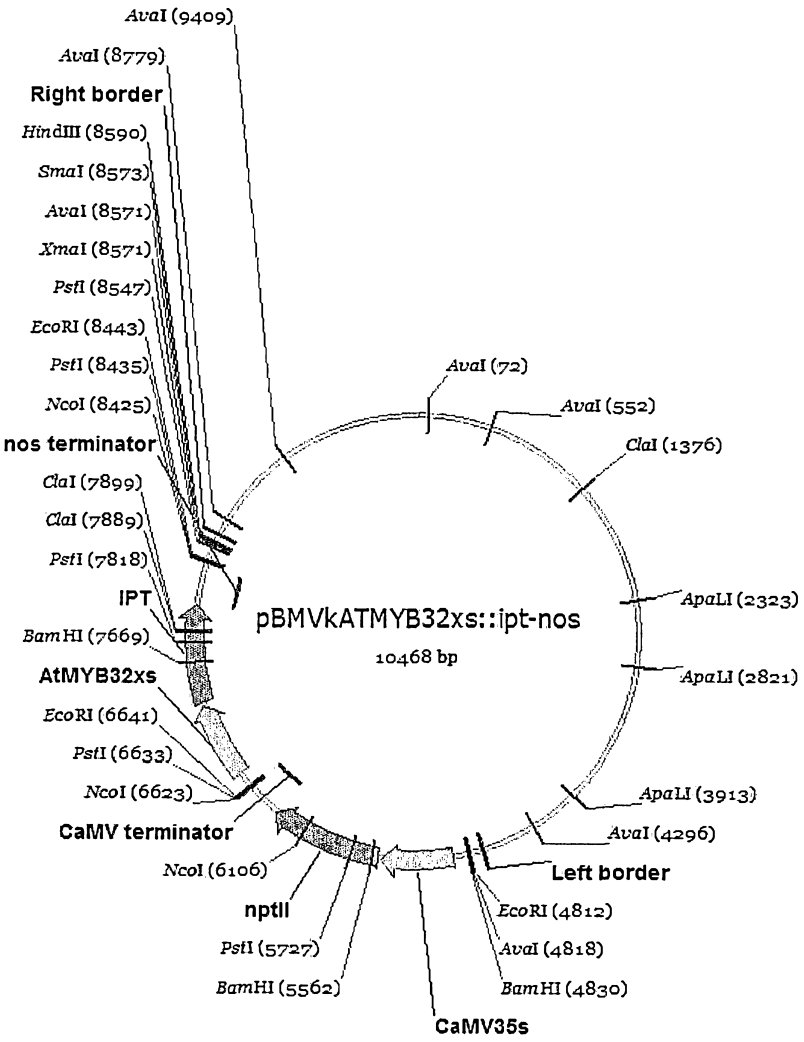
Figure 17.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CaMV terminator
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Figure 18.

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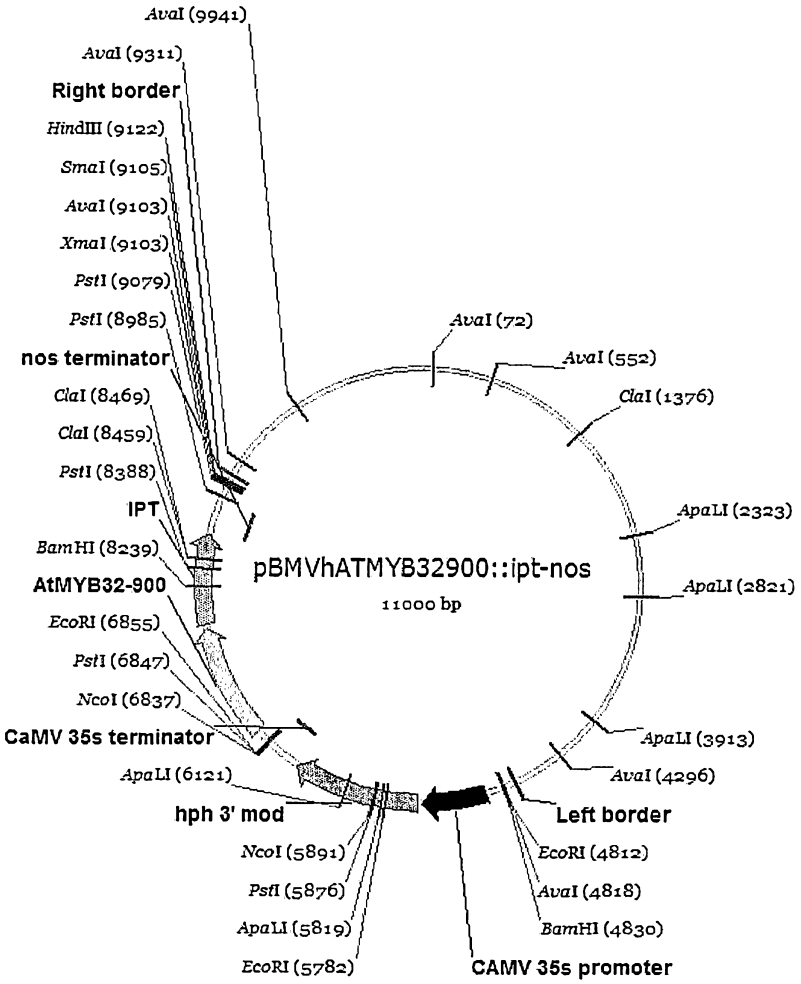
Figure 19.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SmaI (9105)
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XmaI (9103)
PstI (9079)
PstI (8985)
nos terminator
ClaI (8469)
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PstI (8388)
IPT
BamHI (8239)
AtMYB32-900
EcoRI (6855)
PstI (6847)
NcoI (6837)
CaMV 35s terminator
ApaLI (6121)
hph 3' mod
NcoI (5891)
PstI (5876)
ApaLI (5819)
EcoRI (5782)
AvaI (72)
AvaI (552)
ClaI (1376)
ApaLI (2323)
ApaLI (2821)
ApaLI (3913)
AvaI (4296)
Left border
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CAMV 35s promoter
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Figure 20.

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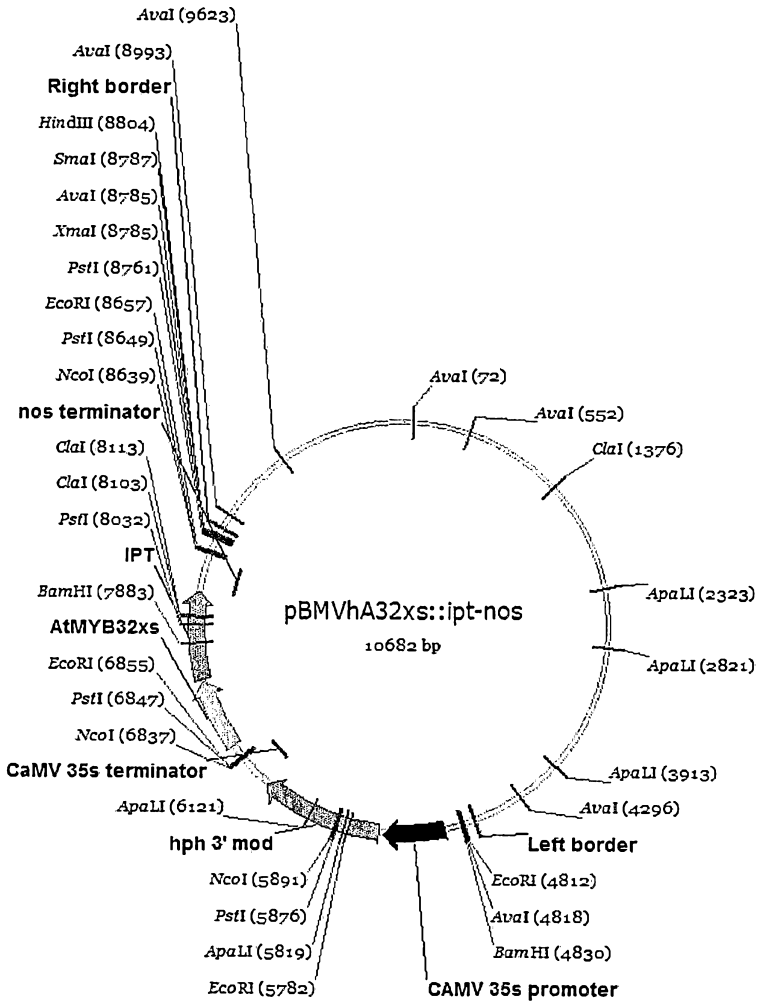
Figure 21.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NcoI (6837)
CaMV 35s terminator
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hph 3' mod
NcoI (5891)
PstI (5876)
ApaLI (5819)
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Figure 22.

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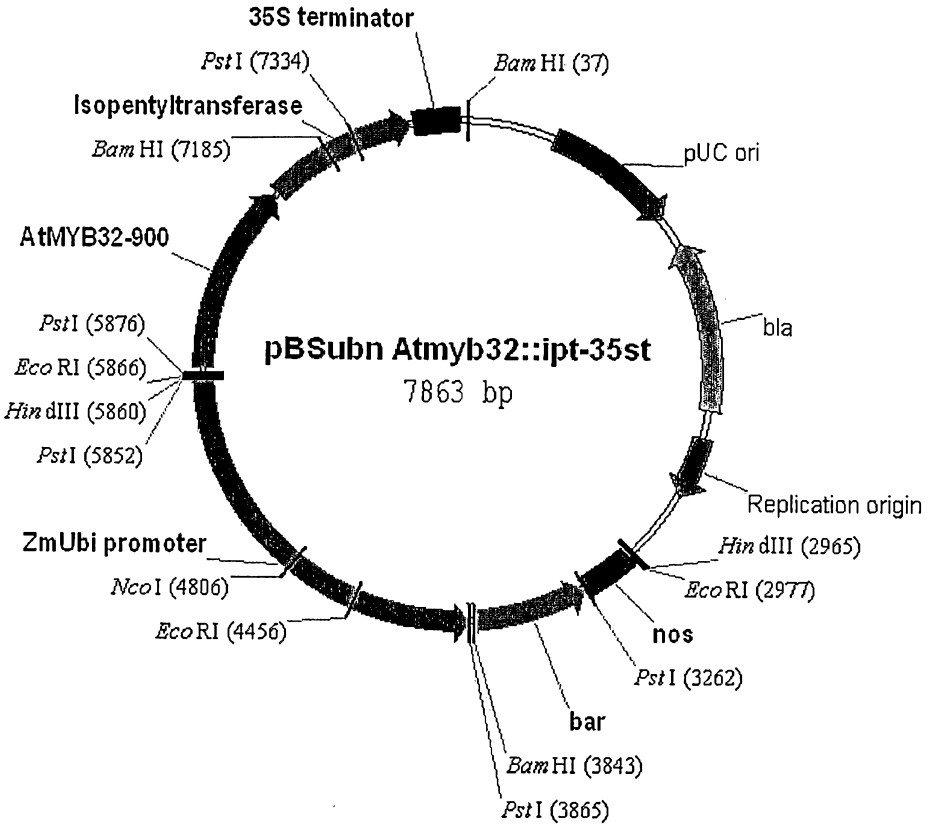
Figure 23.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BamHI (37)
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EcoRI (5866)
HindIII (5860)
PstI (5852)
pBSubn Atmyb32::ipt-35st
7863 bp
bla
Replication origin
ZmUbi promoter
NcoI (4806)
EcoRI (4456)
HindIII (2965)
EcoRI (2977)
nos
PstI (3262)
bar
BamHI (3843)
PstI (3865)

Figure 24.

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 301 taatgaatcg gccaacgcgc ggggagaggc ggtttgcgta ttgggcgctc ttccgcttcc
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Figure 25.

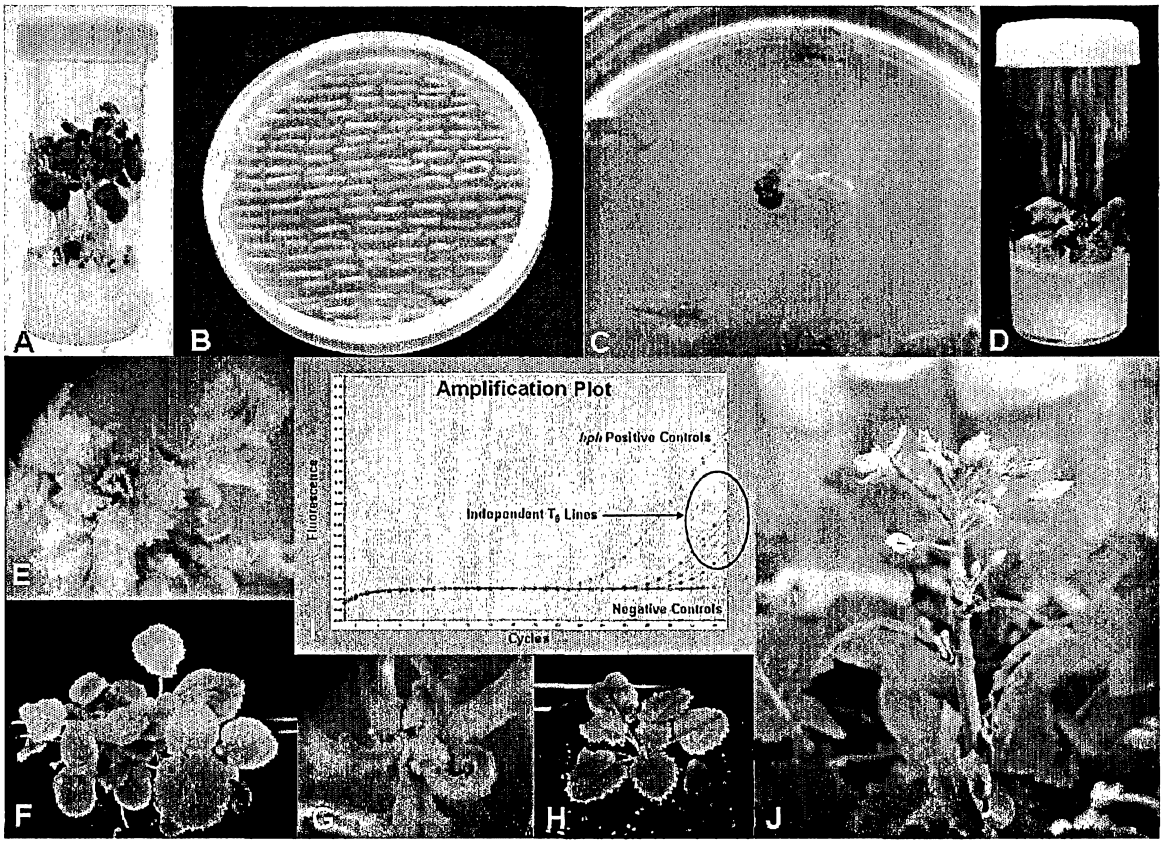

A
B
C
D
E
Amplification Plot
hph Positive Controls
Independant T6 Lines
Negative Controls
Fluorescence
Cycles
F
G
H
J

Figure 26.

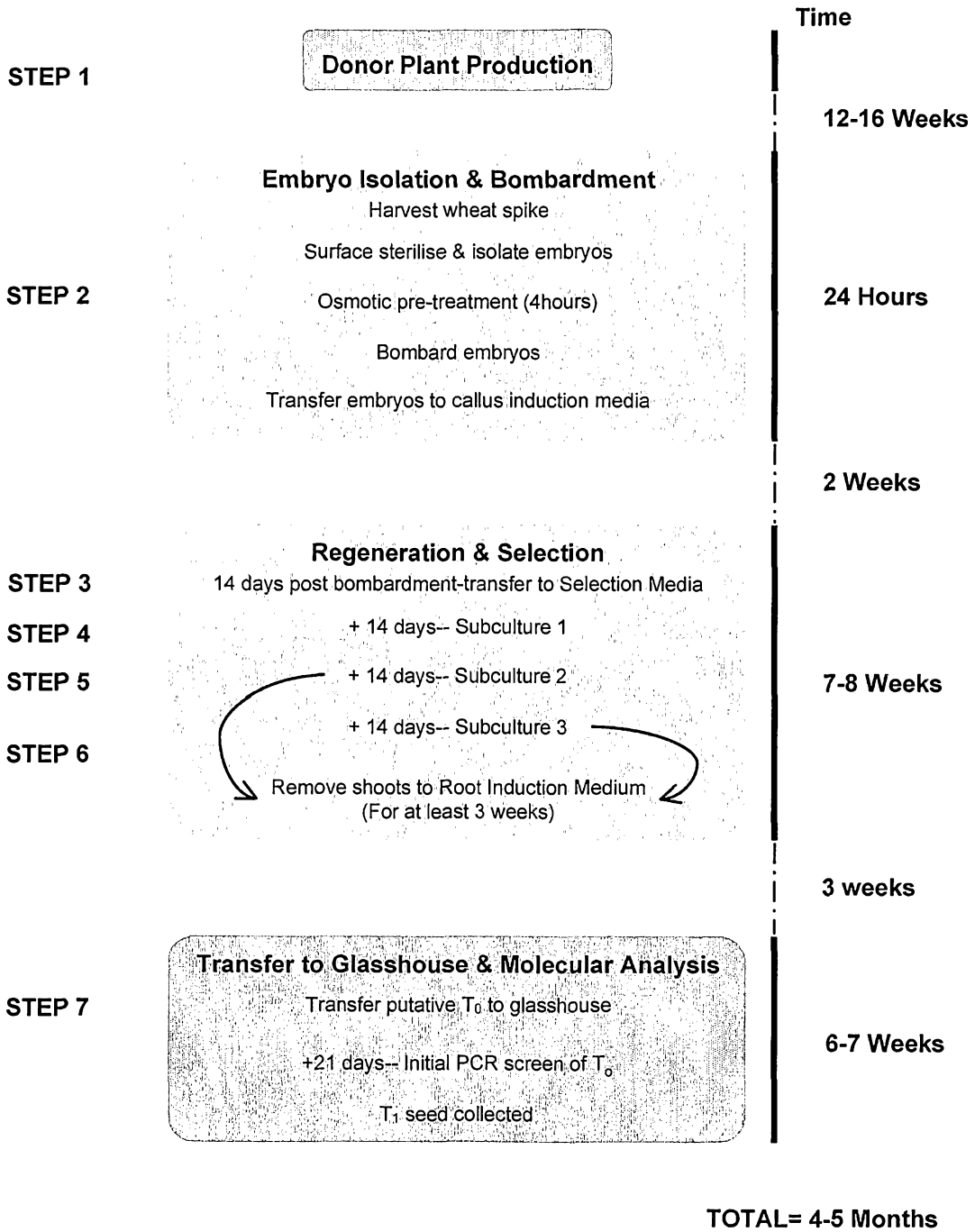

Time
STEP 1
Donor Plant Production
12-16 Weeks
Embryo Isolation & Bombardment
Harvest wheat spike
Surface sterilise & isolate embryos
STEP 2
Osmotic pre-treatment (4hours)
24 Hours
Bombard embryos
Transfer embryos to callus induction media
2 Weeks
Regeneration & Selection
STEP 3
14 days post bombardment-transfer to Selection Media
STEP 4
+ 14 days-- Subculture 1
STEP 5
+ 14 days-- Subculture 2
7-8 Weeks
+ 14 days-- Subculture 3
STEP 6
Remove shoots to Root Induction Medium
(For at least 3 weeks)
3 weeks
Transfer to Glasshouse & Molecular Analysis
STEP 7
Transfer putative T0 to glasshouse
6-7 Weeks
+21 days-- Initial PCR screen of T0
T1 seed collected
TOTAL= 4-5 Months

Figure 27.

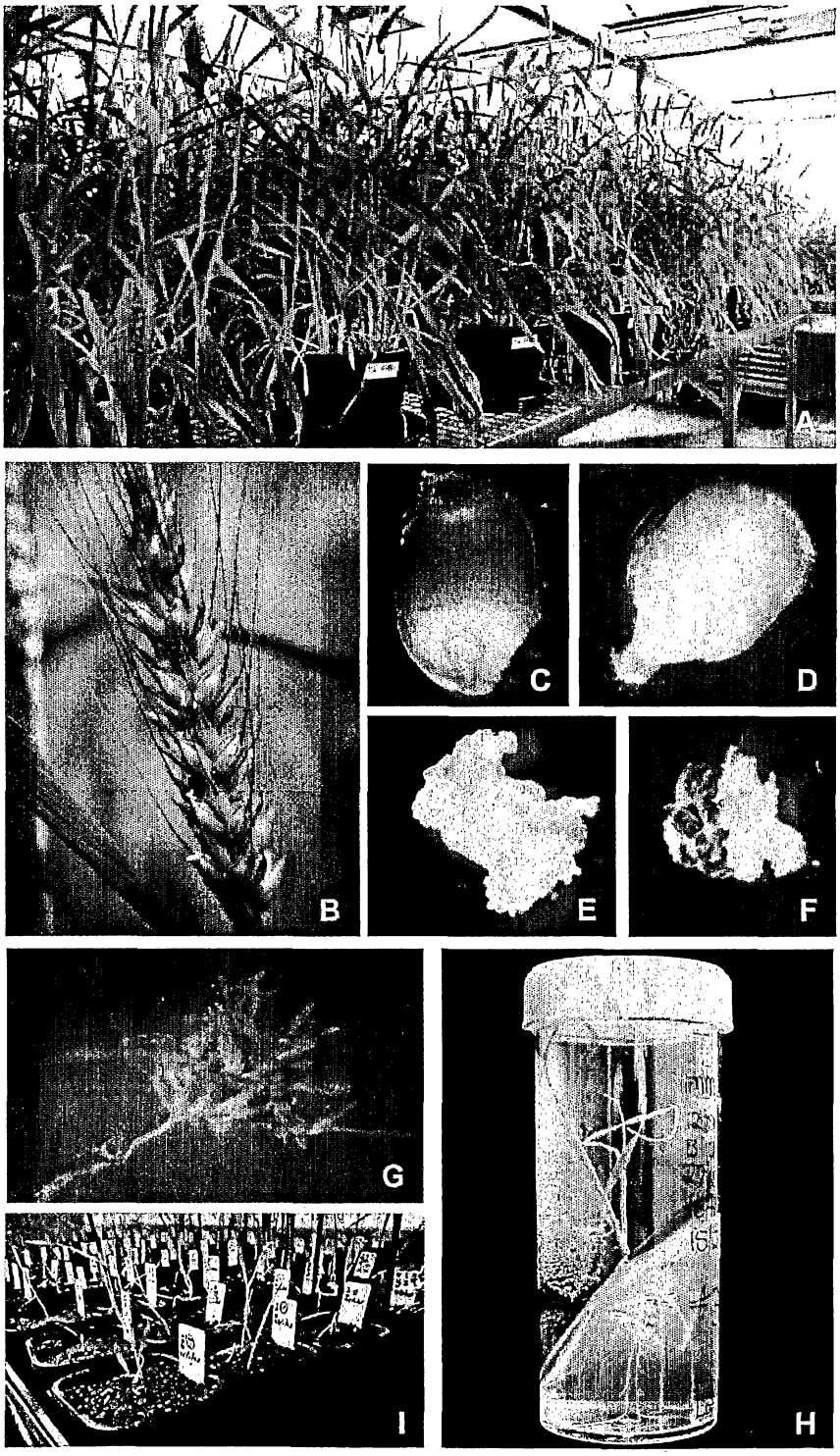


Figure 28.

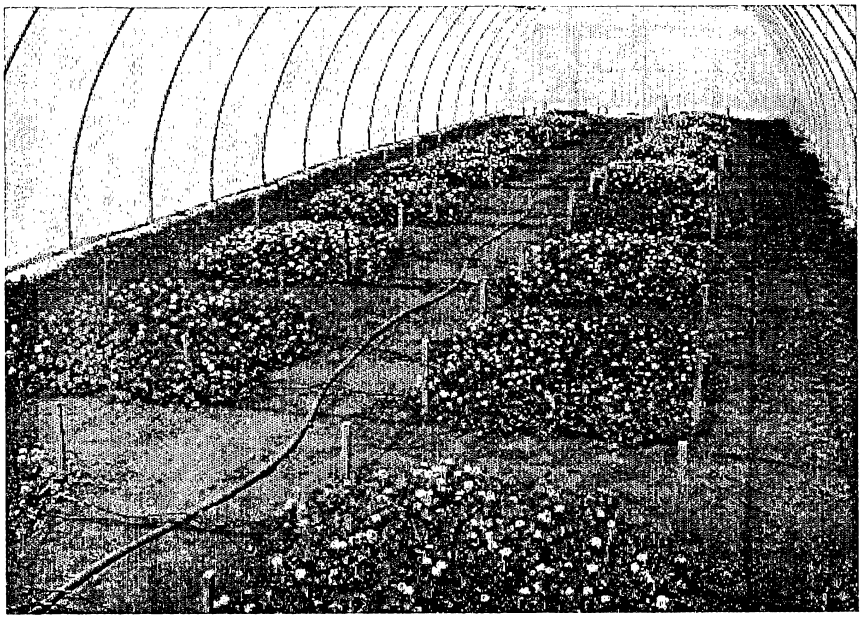
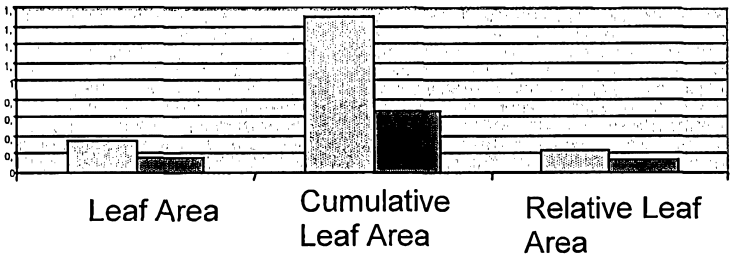
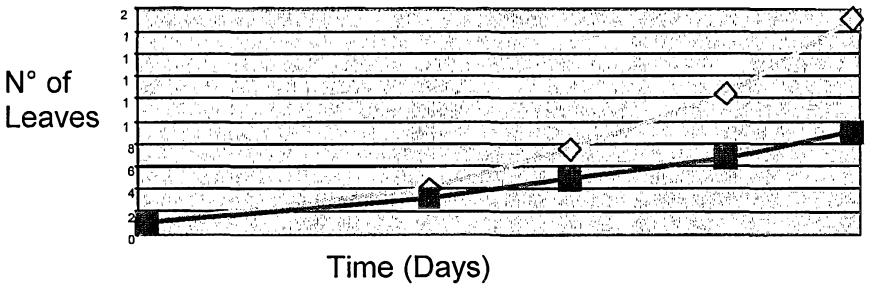


Figure 29.


Leaf Area
Cumulative Leaf Area
Relative Leaf Area


N° of Leaves
Time (Days)

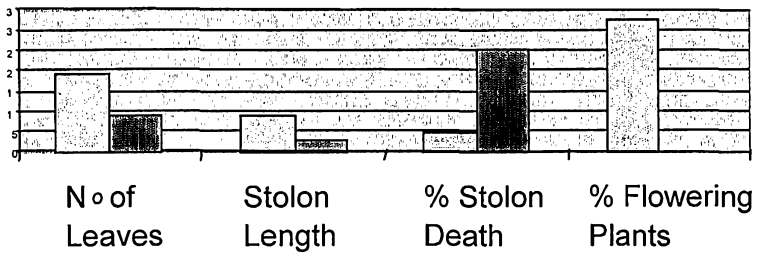
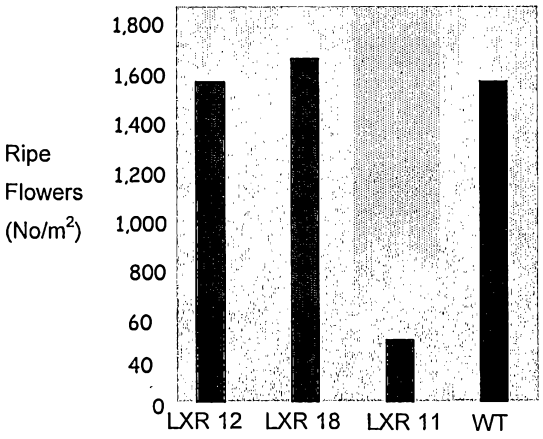

N o of Leaves
Stolon Length
% Stolon Death
% Flowering Plants

Figure 30.


Ripe
Flowers
(No/m2)
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1,400
1,200
1,000
800
60
40
0
LXR 12
LXR 18
LXR 11
WT

Figure 31.

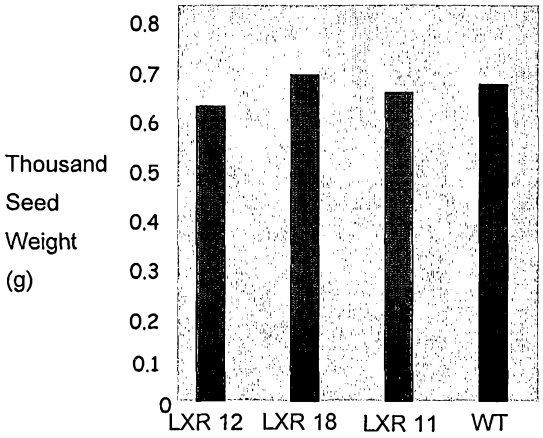


Figure 32.

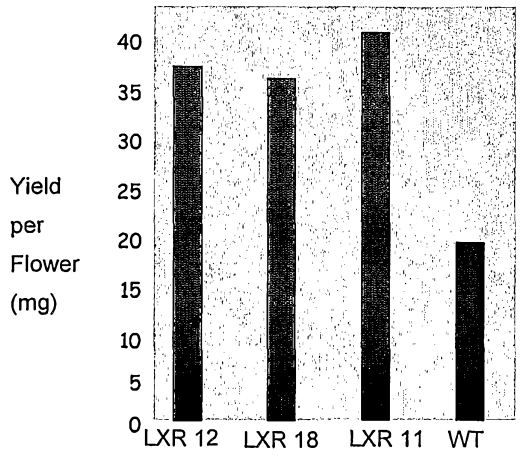


Figure 33.

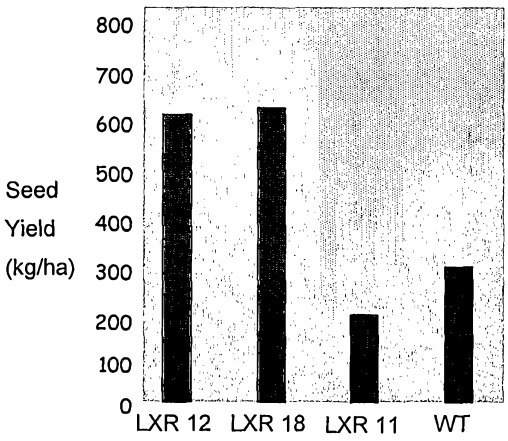

800
700
600
500
400
300
200
100
0
Seed
Yield
(kg/ha)
LXR 12
LXR 18
LXR 11
WT

Figure 34.

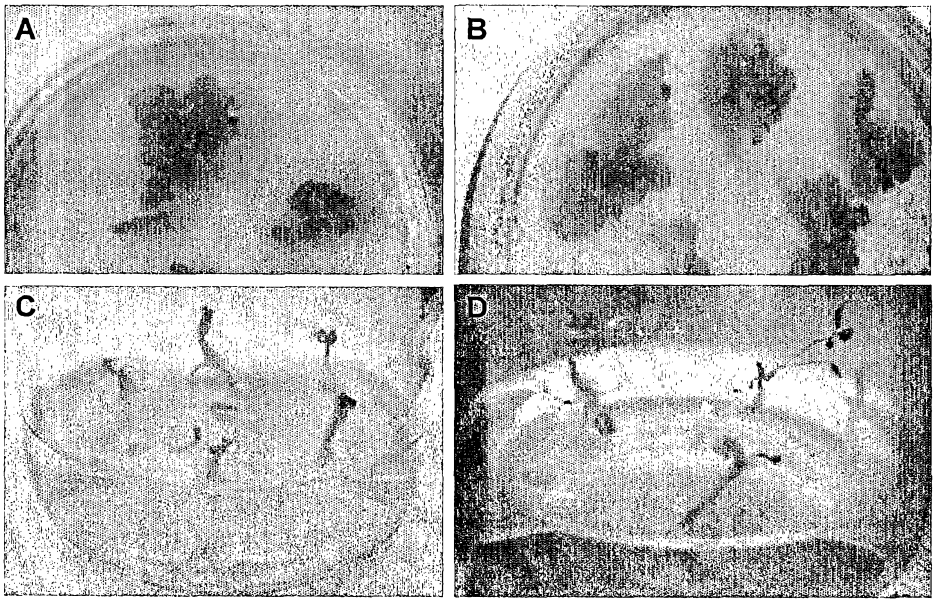
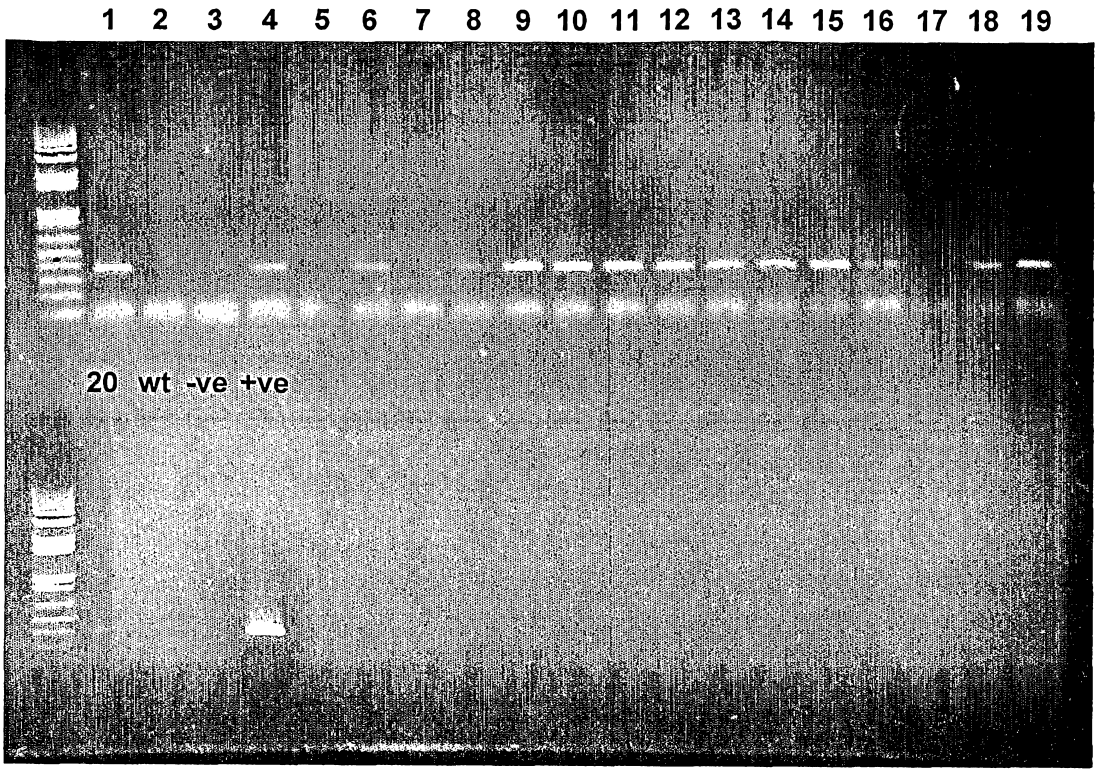


Figure 35

A.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19
20 wt -ve +ve

B.

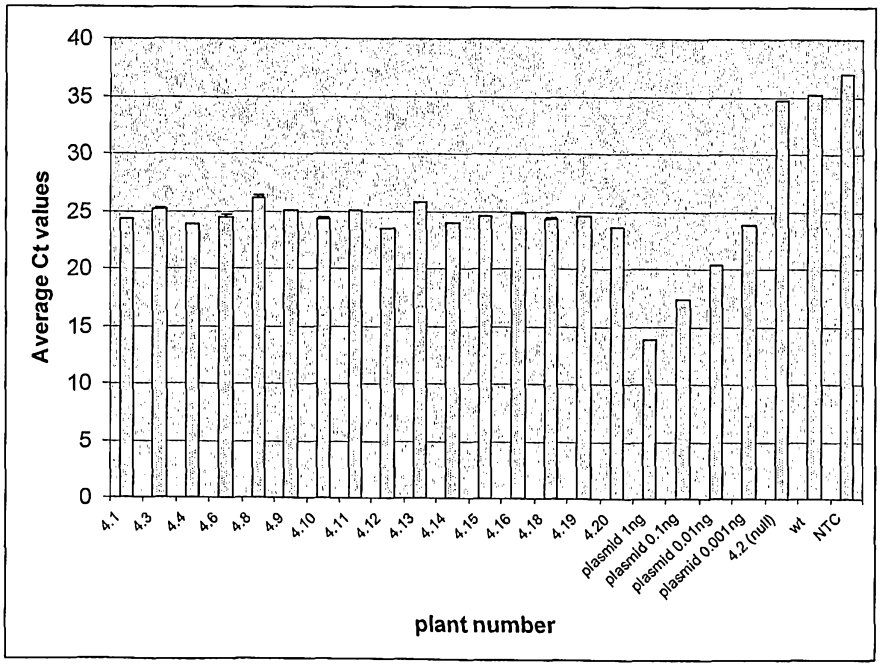

Average Ct values
40
35
30
25
20
15
10
5
0
4.1
4.3
4.4
4.6
4.8
4.9
4.10
4.11
4.12
4.13
4.14
4.15
4.16
4.18
4.19
4.20
plasmid 1ng
plasmid 0.1ng
plasmid 0.01ng
plasmid 0.001ng
4.2 (null)
wt
NTC
plant number

Figure 36

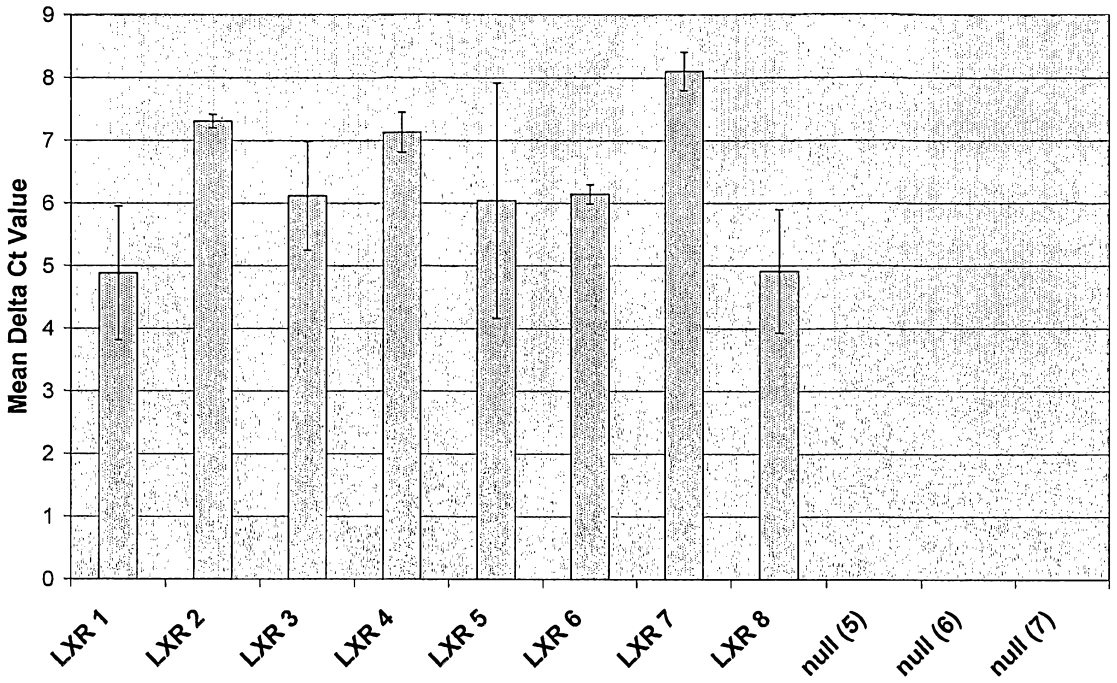

Mean Delta Ct Value
9
8
7
6
5
4
3
2
1
0
LXR 1
LXR 2
LXR 3
LXR 4
LXR 5
LXR 6
LXR 7
LXR 8
null (5)
null (6)
null (7)

Figure 37

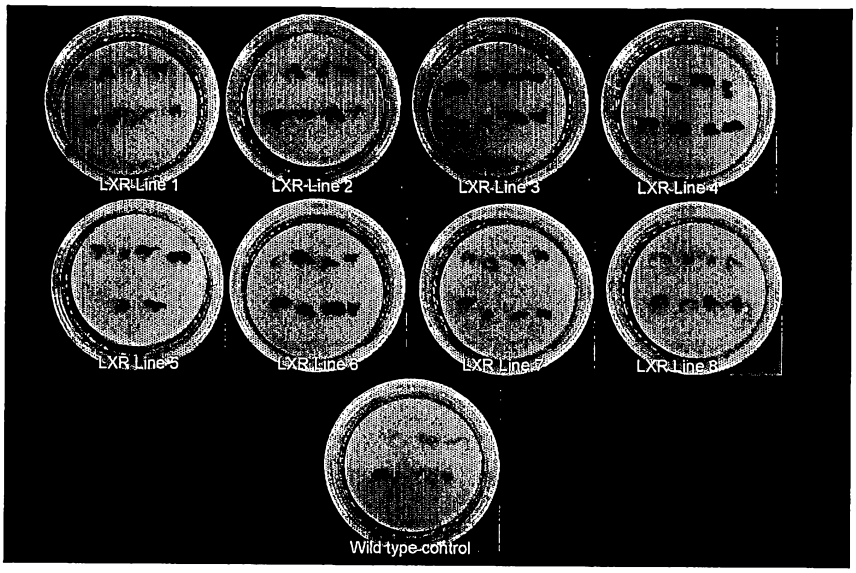

LXR-Line 1
LXR-Line 2
LXR-Line 3
LXR-Line 4
LXR-Line 5
LXR-Line 6
LXR-Line 7
LXR-Line 8
Wild type control

Figure 38

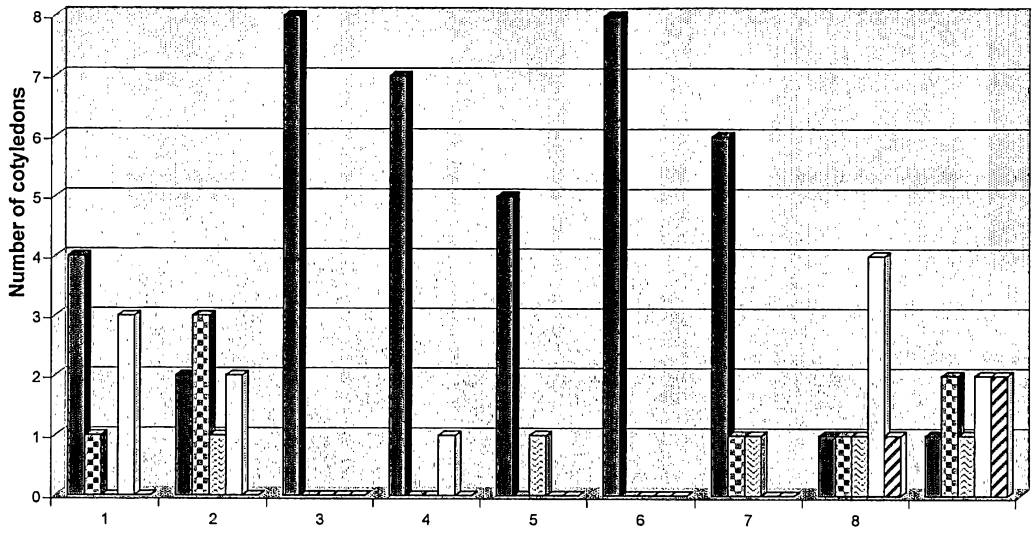


Figure 39

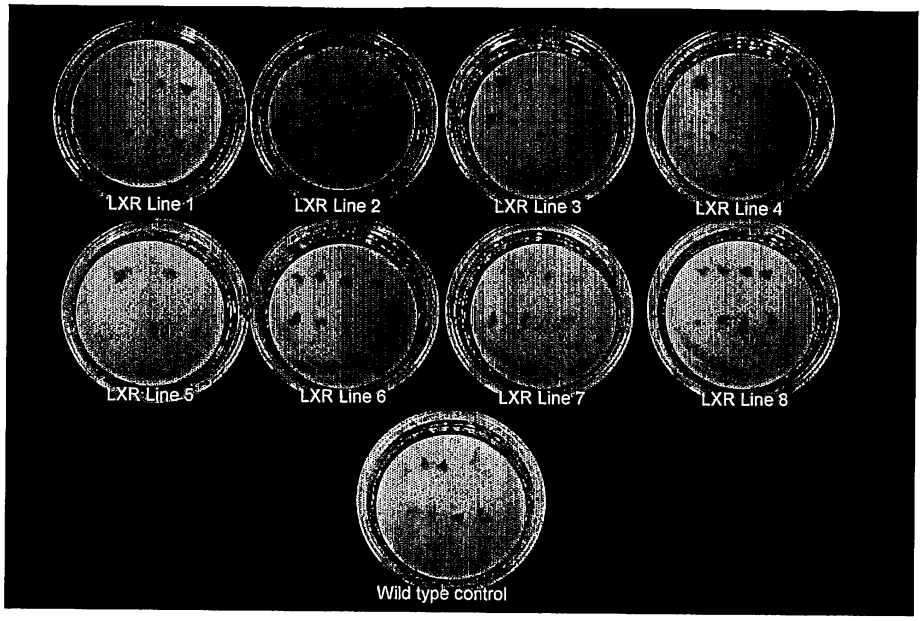

LXR Line 1
LXR Line 2
LXR Line 3
LXR Line 4
LXR Line 5
LXR Line 6
LXR Line 7
LXR Line 8
Wild type control

Figure 40

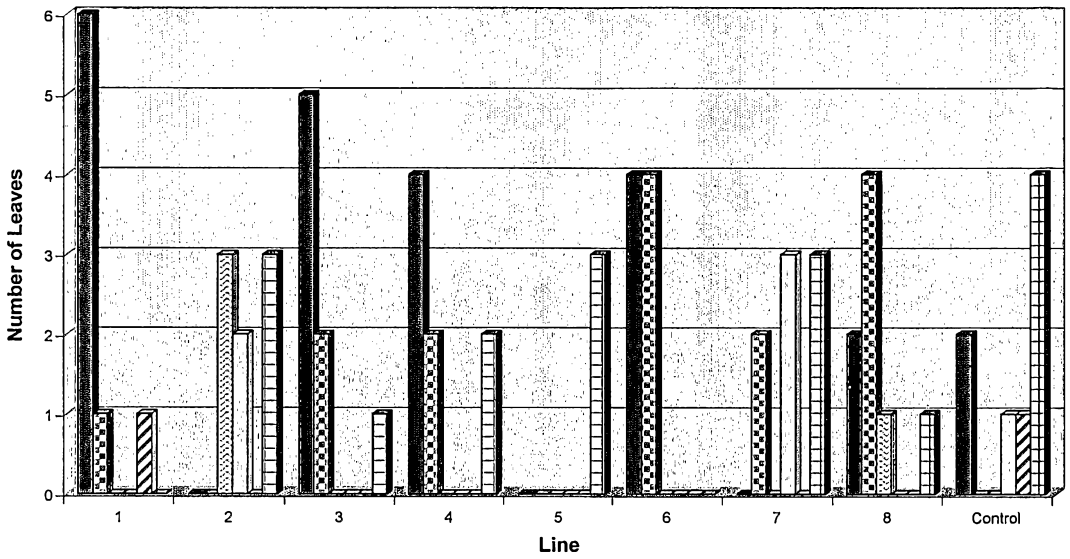


Figure 41

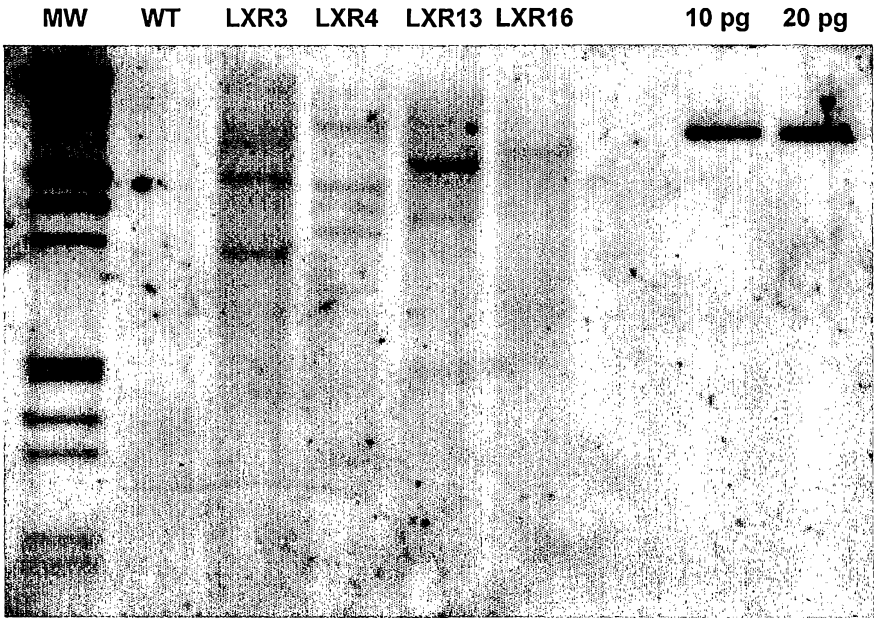


Figure 42

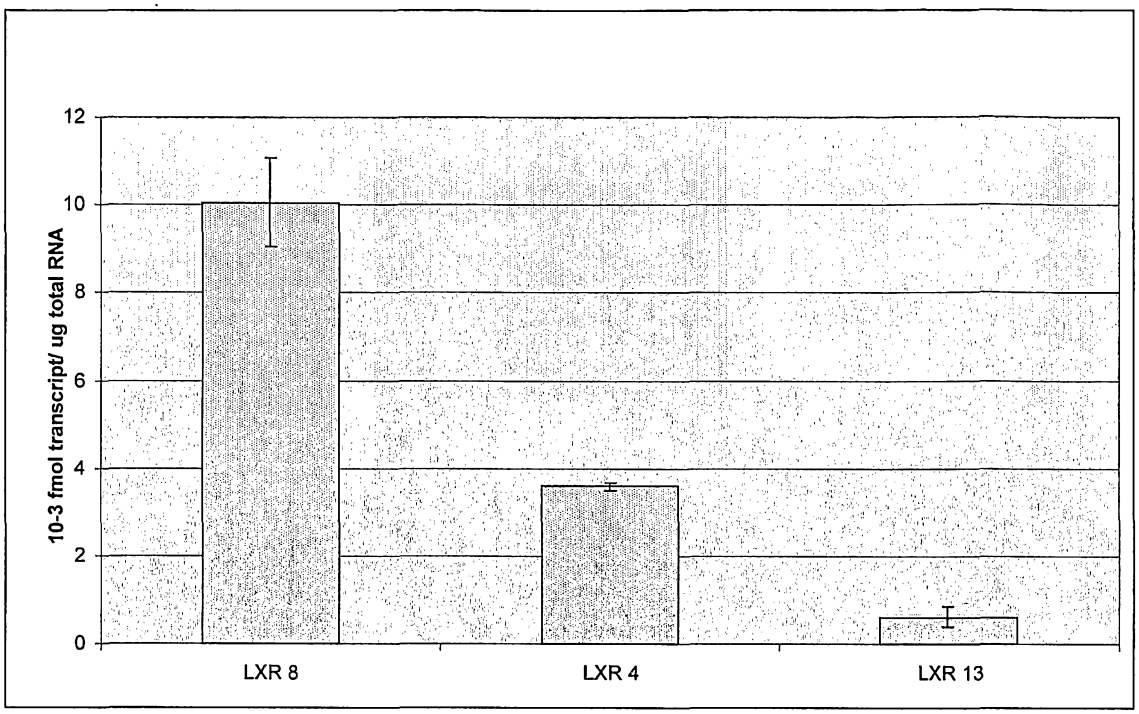


Figure 43

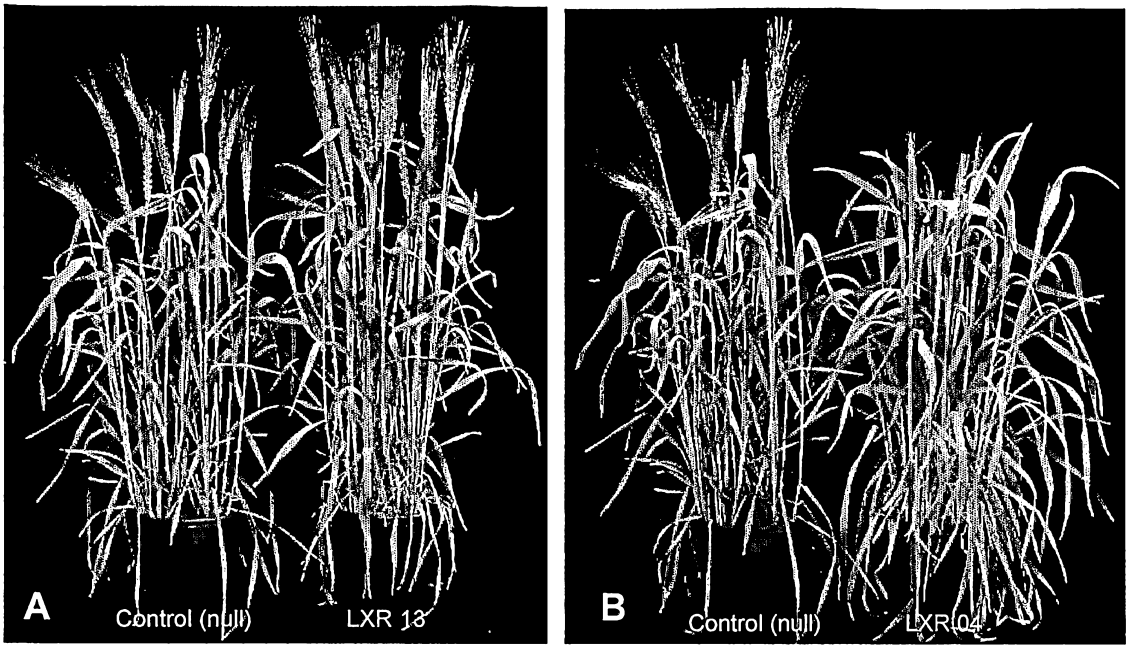


Figure 44

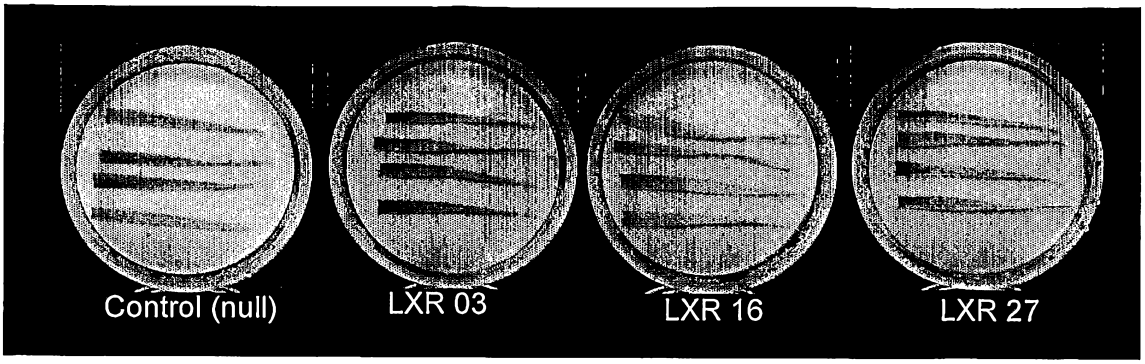


Figure 45

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La Trobe University
Spangenberg, German
Ramage, Carl M
Palviainen, Melissa A
Parish, Roger W
Heazlewood, Joshua

<120> Manipulation of plant senescence using modified promotors

<130> 81381520

<150> US 10/363723
<151> 2001-08-30

<150> US 11/798526
<151> 2007-04-24

<150> PCT/AU01/01092
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<160> 15

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tacttaattt ggtcatttgg atgcccttta caacctcctt accaaactca ctatggcaaa 180

tatatactat tttccattgt aacataaatg tccataattt gaattaaatt cgttgcagta 240

cgaaaccatc caactttgtc caaaaacaaa atccttataa ctatttactt taatgtaaat 300

atatcctcta cttttgtttt tacaacccta gctcaaacaa atttattatt tgcgataaaa 360

aatcatatcg aacaaactcg atgatttttt ttttcttacg ttattaatga aactaaaata 420

tagaaaaaaa caagatgaac caaattttca cctatctaac tacttaaata taatatgatt 480

aaatttggta aagtttgaaa agtttcttta gaaatgtgaa atattgatca cagtttctat 540

tgctaaaatc accaacaaaa cgcatgtcgc cattcataat tatggtttca cacctacaac 600

taggctaata agtaaataag tagacaacta gactcaggtt tgaaaaaacc ataaaagcca 660

tatagcgttt tctcattgaa actgcgaaca cgatcgtgtg aatgttgcag tttctagttt 720

tgatacaaac aaacaaaaac acaatttaat cttagattaa aaagaaaaaa gagaacggag 780

cccactagcc actccttcaa acgtgtctta ccaactctct tctagaaaca aattaggctt 840

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tacttaattt ggtcatttgg atgcccttta caacctcctt accaaactca ttgatcacag 180

tttctattgc taaaatcacc aacaaaacgc atgtcgccat tcataattat ggtttcacac 240

ctacaactag gctaataagt aaataagtag acaactagac tcaggtttga aaaaaccata 300

aaagccatat agcgttttct cattgaaact gcgaacacga tcgtgtgaat gttgcagttt 360

ctagttttga tacaaacaaa caaaaacaca atttaatctt agattaaaaa gaaaaaagag 420

aacggagccc actagccact ccttcaaacg tgtcttacca actctcttct agaaacaaat 480

taggcttcac cttcctcttc caacctctct ctctctctct ctctcttttt ctcaaaccat 540

ctctccataa agccctaatt tcttcatcac aagaatcaga agaagaaa 588

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<213> Arabidopsis thaliana

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ctacaactag gctaataagt aaataagtag acaactagac tcaggtttga aaaaaccata 180

aaagccatat agcgttttct cattgaaact gcgaacacga tcgtgtgaat gttgcagttt 240

ctagttttga tacaaacaaa caaaaacaca atttaatctt agattaaaaa gaaaaaagag 300

aacggagccc actagccact ccttcaaacg tgtcttacca actctcttct agaaacaaat 360

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aaaaaaccat aaaagccata tagcgttttc tcattgaaac tgcgaacacg atcgtgtgaa 180

tgttgcagtt tctagttttg atacaaacaa acaaaaacac aatttaatct tagattaaaa 240

agaaaaaaga gaacggagcc cactagccac tccttcaaac gtgtcttacc aactctcttc 300

tagaaacaaa ttaggcttca ccttcctctt ccaacctctc tctctctctc tctctctttt 360

tctcaaacca tctctccata aagccctaat ttcttcatca caagaatcag aagaagaaa 419

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ctatcaaccg gaagcggacg accaacagtg gaagaactga aaggaacgac gcgtctctac 180

cttgatgatc ggcctctggt ggagggtatc atcgcagcca agcaagctca tcataggctg 240

atcgaggagg tgtataatca tgaggccaac ggcgggctta ttcttgaggg aggatccacc 300

tcgttgctca actgcatggc gcgaaacagc tattggagtg cagattttcg ttggcatatt 360

attcgccaca agttacccga ccaagagacc ttcatgaaag cggccaaggc cagagttaag 420

cagatgttgc accccgctgc aggccattct attattcaag agttggttta tctttggaat 480

gaacctcggc tgaggcccat tctgaaagag atcgatggat atcgatatgc catgttgttt 540

gctagccaga accagatcac ggcagatatg ctattgcagc ttgacgcaaa tatggaaggt 600

aagttgatta atgggatcgc tcaggagtat ttcatccatg cgcgccaaca ggaacagaaa 660

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tag 723

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<211> 240
<212> PRT
<213> Agrobacterium tumefaciens

<400> 6

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20 25 30

Asp Arg Val Gln Cys Cys Pro Gln Leu Ser Thr Gly Ser Gly Arg Pro
35 40 45

Thr Val Glu Glu Leu Lys Gly Thr Thr Arg Leu Tyr Leu Asp Asp Arg
50 55 60

Pro Leu Val Glu Gly Ile Ile Ala Ala Lys Gln Ala His His Arg Leu
65 70 75 80

Ile Glu Glu Val Tyr Asn His Glu Ala Asn Gly Gly Leu Ile Leu Glu
85 90 95

Gly Gly Ser Thr Ser Leu Leu Asn Cys Met Ala Arg Asn Ser Tyr Trp
100 105 110

Ser Ala Asp Phe Arg Trp His Ile Ile Arg His Lys Leu Pro Asp Gln
115 120 125

Glu Thr Phe Met Lys Ala Ala Lys Ala Arg Val Lys Gln Met Leu His
130 135 140

Pro Ala Ala Gly His Ser Ile Ile Gln Glu Leu Val Tyr Leu Trp Asn
145 150 155 160

Glu Pro Arg Leu Arg Pro Ile Leu Lys Glu Ile Asp Gly Tyr Arg Tyr
165 170 175

Ala Met Leu Phe Ala Ser Gln Asn Gln Ile Thr Ala Asp Met Leu Leu
180 185 190

Gln Leu Asp Ala Asn Met Glu Gly Lys Leu Ile Asn Gly Ile Ala Gln
195 200 205

Glu Tyr Phe Ile His Ala Arg Gln Gln Glu Gln Lys Phe Pro Gln Val
210 215 220

Asn Ala Ala Ala Phe Asp Gly Phe Glu Gly His Pro Phe Gly Met Tyr
225 230 235 240

<210> 7
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<212> DNA
<213> Lotus japonicus

<400> 7
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acagggacag gaaagtcaaa gctctccatt gacctcgcca cctgtttccc ctcagaaatc 180

atcaactccg acaagattca aatctacgac ggcctcgaca tcgtcaccaa caaaatctcc 240

aaggaagaac aacgtggaat cccccaccac ctcctcggaa ctcaaaaccc taacacagac 300

ttcaccgccg gcgatttcag tgactgttcc accgccgcca ttgacgcaat cacaagccgc 360

gaccaccttc cgatcatcgc cggaggttcg aactcctacc tggaggcgtt aatcgacgac 420

gacgactaca aattccgatc gaggtacgac ttctgctgcc tctgggtcga cgtggcaatg 480

ccggtgctgg actcatacgt ggcggcgcgt gtggatcaga tgctccggag cggaatggtg 540

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aggaggcaat tggggaagat tcagaggctg aggaatgtga agaggtggga gattcaccgt 780

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1 5 10 15

Val Pro Cys Ser Gly Lys Lys Leu Ser Met Arg Gln Ile Gln Lys Glu
20 25 30

Lys Val Val Leu Val Met Gly Ala Thr Gly Thr Gly Lys Ser Lys Leu
35 40 45

Ser Ile Asp Leu Ala Thr Cys Phe Pro Ser Glu Ile Ile Asn Ser Asp
50 55 60

Lys Ile Gln Ile Tyr Asp Gly Leu Asp Ile Val Thr Asn Lys Ile Ser
65 70 75 80

Lys Glu Glu Gln Arg Gly Ile Pro His His Leu Leu Gly Thr Gln Asn
85 90 95

Pro Asn Thr Asp Phe Thr Ala Gly Asp Phe Ser Asp Cys Ser Thr Ala
100 105 110

Ala Ile Asp Ala Ile Thr Ser Arg Asp His Leu Pro Ile Ile Ala Gly
115 120 125

Gly Ser Asn Ser Tyr Leu Glu Ala Leu Ile Asp Asp Asp Asp Tyr Lys
130 135 140

Phe Arg Ser Arg Tyr Asp Phe Cys Cys Leu Trp Val Asp Val Ala Met
145 150 155 160

Pro Val Leu Asp Ser Tyr Val Ala Ala Arg Val Asp Gln Met Leu Arg
165 170 175

Ser Gly Met Val Glu Glu Leu Arg Pro Phe Phe Asn Ala Asn Gly Asp
180 185 190

Tyr Ser Arg Gly Ile Arg Arg Ala Ile Gly Val Pro Glu Phe Asp Glu
195 200 205

Tyr Phe Arg Arg Glu Gly Phe Ala Asp Glu Glu Thr Arg Lys Leu Leu
210 215 220

Leu Glu Arg Ala Val Arg Glu Met Lys Val Asn Thr Cys Lys Leu Ala
225 230 235 240

Arg Arg Gln Leu Gly Lys Ile Gln Arg Leu Arg Asn Val Lys Arg Trp
245 250 255

Glu Ile His Arg Val Asp Ala Thr Pro Val Phe Trp Lys Arg Gly Glu
260 265 270

Glu Ala Asp Glu Ala Trp Arg Lys Val Val Ala Glu Pro Ser Ala Met
275 280 285

Ile Val Ala Gln Phe Leu Tyr Lys Ala Lys Ser Asp Val Asn Val Val
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Ser Gly Gly Phe Arg Val Pro Ala Gly Ser Thr Glu Ser Val Met Ala
305 310 315 320

Ala Ala Thr Cys

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atacccggcc ccacacaaaa aaacaaaaac aaaatcatag taataatggg tgcaacaggt 240

tcaggaaaat caaaactctc aatagacctc gtcacacgtc actatccttt ttccgaaatc 300

attaactccg acaaaatcca aattaccaaa ggtttaaaca taaccacaaa caaaatcact 360

gtacccgacc gacgtggcgt agttcatcat ttactcggcg agattgaccc cgactttaac 420

ttttctcctt ctcatttccg gtcaattgct ggtcaacgca ttaactccat tattaatcgc 480

cataaactcc cattcctcgt tggtgggtcc aactcatata tctacgcttt attaacaaac 540

cggttcgacc cggattttaa ccctgattca aacccggttc attttatatc caacgagtta 600

cgctacaact gttgttttat ttgggtcgat gtattaaacc cggttttgaa tgagtatttg 660

gataaacggg tcgatgagat gatgaactcg ggtatgtatg aagaactgga acagtttttt 720

aaagaaaaca ggttttcgga tccgggtttg gaaccgggtc gggccaccgg gttgaggaaa 780

gcgatagggg taccggaaat ggagaggtat tttaagaaga gctgtacgta tgaggaagca 840

gtgagggaaa taaaagaaaa cacgtggcgg ttagcgaaga agcagatgtg gaagatccaa 900

cggttgagag aagcagggtg ggacctacaa agagtagatg ccacggaggc atttgtggag 960

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<400> 10

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20 25 30

Tyr Asn His Lys His Leu Lys Phe Leu Thr Asn Pro Thr Thr Arg Val
35 40 45

Leu Arg Arg Asn Met Ser Ser Ser Thr Val Val Thr Ile Pro Gly Pro
50 55 60

Thr Gln Lys Asn Lys Asn Lys Ile Ile Val Ile Met Gly Ala Thr Gly
65 70 75 80

Ser Gly Lys Ser Lys Leu Ser Ile Asp Leu Val Thr Arg His Tyr Pro
85 90 95

Phe Ser Glu Ile Ile Asn Ser Asp Lys Ile Gln Ile Thr Lys Gly Leu
100 105 110

Asn Ile Thr Thr Asn Lys Ile Thr Val Pro Asp Arg Arg Gly Val Val
115 120 125

His His Leu Leu Gly Glu Ile Asp Pro Asp Phe Asn Phe Ser Pro Ser
130 135 140

His Phe Arg Ser Ile Ala Gly Gln Arg Ile Asn Ser Ile Ile Asn Arg
145 150 155 160

His Lys Leu Pro Phe Leu Val Gly Gly Ser Asn Ser Tyr Ile Tyr Ala
165 170 175

Leu Leu Thr Asn Arg Phe Asp Pro Asp Phe Asn Pro Asp Ser Asn Pro
180 185 190

Val His Phe Ile Ser Asn Glu Leu Arg Tyr Asn Cys Cys Phe Ile Trp
195 200 205

Val Asp Val Leu Asn Pro Val Leu Asn Glu Tyr Leu Asp Lys Arg Val
210 215 220

Asp Glu Met Met Asn Ser Gly Met Tyr Glu Glu Leu Glu Gln Phe Phe

225 230 235 240

Lys Glu Asn Arg Phe Ser Asp Pro Gly Leu Glu Pro Gly Arg Ala Thr
245 250 255

Gly Leu Arg Lys Ala Ile Gly Val Pro Glu Met Glu Arg Tyr Phe Lys
260 265 270

Lys Ser Cys Thr Tyr Glu Glu Ala Val Arg Glu Ile Lys Glu Asn Thr
275 280 285

Trp Arg Leu Ala Lys Lys Gln Met Trp Lys Ile Gln Arg Leu Arg Glu
290 295 300

Ala Gly Trp Asp Leu Gln Arg Val Asp Ala Thr Glu Ala Phe Val Glu
305 310 315 320

Ala Met Ser Asn Lys Lys Glu Lys Gly Ile Ile Trp Glu Lys Gln Val
325 330 335

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<223> Synthetic construct

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atcaaaactc atttgagtta atgaggtaaa gagaaaatga gcaaaagcac aaacacgcta 180

agtgccggcc gtccgagcgc acgcagcagc aaggctgcaa cgttggccag cctggcagac 240

acgccagcca tgaagcgggt caactttcag ttgccggcgg aggatcacac caagctgaag 300

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ctaccagagt aaatgagcaa atgaataaat gagtagatga attttagcgg ctaaaggagg 420

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aacgggcggt tggccaggcg taagcggctg ggttgtctgc cggccctgca atggcactgg 540

aacccccaag cccgaggaat cggcgtgacg gtcgcaaacc atccggcccg gtacaaatcg 600

gcgcggcgct gggtgatgac ctggtggaga agttgaaggc cgcgcaggcc gcccagcggc 660

aacgcatcga ggcagaagca cgccccggtg aatcgtggca agcggccgct gatcgaatcc 720

gcaaagaatc ccggcaaccg ccggcagccg gtgcgccgtc gattaggaag ccgcccaagg 780

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